Relevance of anti-myelin antibodies in Multiple Sclerosis

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Relevance of anti-myelin antibodies in Multiple Sclerosis

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Voor mijn ouders

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Abbreviations

APC	Antigen presenting cell
BBB	Blood brain barrier
BCR	B cell receptor
CDMS	Clinically definite MS
CIS	Clinically isolated syndrome
CNP	2'-3'-cyclic nucleotide 3'-phosphodiesterase
CNS	Central nervous system
CR	Complement receptor(s)
CSF	Cerebrospinal fluid
CVF	Cobra venom factor
EAE	Experimental allergic (autoimmune) encephalomyelitis
EM	Electron microscopy
FcγR	Fcγ receptor(s)
FcRγ chain	FcRγ chain
FcRγ$^{-/-}$ mice	FcRγ chain knockout mice
GalC	galactosylceramide
Ig	Immunoglobulin
ITAM	Intracellular immunoreceptor tyrosine-based activation motif
ITIM	Intracellular immunoreceptor tyrosine-based inhibitory motif
MAC	Membrane attack complex, terminal complex of complement
MAG	Myelin associated glycoprotein
MBP	Myelin basic protein
MOG	Myelin oligodendrocyte glycoprotein
MS	Multiple Sclerosis
OCB	Oligoclonal immunoglobulin bands
OIND	Other inflammatory neurological disease
OSP	Oligodendrocyte specific protein
PLP	Proteolipid protein
PPMS	Primary progressive MS
rMOG	Recombinant MOG, derived from rat (rrMOG), mouse (rmMOG) or human (rhMOG) sequence, representing amino acids 1-125
RRMS	Relapsing remitting MS
SPMS	Secondary progressive MS
T_{reg}	Regulatory T cell
Wt mice	Wild-type mice

Chapter 1

General Introduction

Index

1. Multiple sclerosis

1.1 . Clinical features, epidemiology and aetiology

Multiple sclerosis (MS) is a chronic disabling disease of the central nervous system (CNS), mostly affecting young adults. Clinical signs are heterogeneous, including a wide variety of motor deficits such as muscle weakness, tremor and paralysis, often accompanied by sensory deficits, such as visual impairment. In eighty-five percent of patients MS clinically presents between the age of 20 and 40 as episodes of loss of motor and/or sensory function (relapses), followed by periods of complete or incomplete recovery (remission). The relapsing remitting (RRMS) phase is usually followed by the secondary progressive phase (SPMS) where motor and sensory deficits gradually accumulate without periods of remission (Lublin and Reingold, 1996). In fifty percent of RRMS patients this occurs within 10 years after onset of disease, and within 25 years after onset over ninety percent of patients that started with RRMS have developed SPMS. The RR-SP disease course occurs in women and men in a ratio of 2:1. In fifteen percent of MS patients, disease takes a primary progressive course (PPMS) without distinct periods of recovery. Disease onset in these patients is generally later and the rate of deterioration is higher than in SPMS. Intriguingly, the women to men ratio in PPMS patients is 1,3:1 suggesting that underlying pathogenetic mechanisms may be somewhat different than in RR-SPMS (Cottrell *et al.*, 1999).

The aetiology of MS is poorly understood. Disease preferentially affects individuals of Northern European descent with geographical differences in disease incidence ranging from 1:20.000 in equatorial areas to 1:1000 in the Netherlands, the United Kingdom, Southern Scandinavia and Southern Canada (Rosati, 2001). This suggests that both genetic and environmental factors contribute to MS susceptibility.

A considerable number of genetic polymorphisms, mostly in genes related to the immune system, have been associated with MS susceptibility (van Veen *et al.*, 2001; van Veen *et al.*, 2002; van Veen *et al.*, 2003). However, associations are usually not very strong and often not specific for MS. The only gene unambiguously associated with MS is HLA-DR. Carriers of the HLA-DRB1*1501 haplotype have an increased risk of developing MS. However, even in this case the relative risk is not higher than 2-4, theoretically increasing the chance of developing MS from maximally 1:1000 to maximally 1:250 (Dyment *et al.*, 2004). Nevertheless, the concordance of MS in monozygotic (female) twins is thirty percent, compared to 2-3 percent in dizygotic twins, emphasizing the relevance of genetic background (Dyment *et al.*, 2004). It is obvious that the genetics of MS are complex, and linkage studies including a large number of genes and a large number of subjects are required to identify genetic patterns of susceptibility (Hooper-van Veen, 2003).

Environmental factors implicated in MS pathogenesis include geographical differences in exposure to sunlight (vitamin D) and infections, particularly viral infections in childhood (Marrie, 2004). A large number of viral infections have been associated with MS, including measles virus and herpes viruses but again no single culprit has been identified (Granieri *et al.*, 2001; Stuve *et al.*, 2004).

1.2. Myelin and demyelination

Neuropathologically, MS is characterized by focal areas of myelin loss (plaques), where myelin is eventually replaced by an astrogliotic scar ('sclerosis').

Myelin is composed of oligodendrocyte membranes tightly wrapped around axons, forming a layer of electrical insulation (Figure 1A). Myelin sheaths, together with the nodes of Ranvier - short sections of unmyelinated axon that separate different segments of the myelin sheath - form a prerequisite for rapid conduction of nerve impulses over relatively long distances. In addition, myelin supports axonal growth and axonal survival. Adjacent myelin sheaths on one axon usually belong to different oligodendrocytes, whereas processes from a single oligodendrocyte form myelin sheaths around a varying number of axons, ranging from 1 to 40, depending on the area of the CNS (Baumann and Pham-Dinh, 2001). The high lipid content of myelin causes the white appearance of myelinated areas in the brain, hence called CNS white matter. The myelin content in the cerebral cortex and the spinal cord medulla - the CNS grey matter - is much lower, and the myelin sheaths that are present are thinner than in white matter.

Seventy percent of myelin dry weight consists of lipids, including cholesterol, phospholipids, glycolipids and glycosphingolipids, particularly galactosylceramides (GalC). Myelin proteins comprise thirty percent of myelin dry weight. In contrast to myelin lipids, most of the proteins are myelin specific. Although the function of some myelin proteins has been studied using specific knockout mice, the role of most myelin proteins remains obscure (reviewed by Baumann and Pham-Dinh, 2001).

Proteolipid protein (PLP, 50% of myelin protein) and myelin basic protein (MBP, 30% of myelin protein) are two major myelin proteins in the CNS. PLP is a transmembrane protein with loops extending into the cytoplasmic interface and the extracellular face of the oligodendrocyte membranes (Figure 1B). PLP is thought to be important for stabilizing membrane layers to form compact myelin. MBP is present on the cytoplasmic interface of the oligodendrocyte membranes (Figure 1B) and exists in a number of isoforms. 'Shiverer' mice have a large deletion in the MBP gene and are unable to form dense myelin sheaths, demonstrating that MBP is important for myelin compaction. Four percent of CNS myelin protein consists of 2'-3'-cyclic nucleotide 3'-phosphodiesterase (CNP), a protein with unknown function. Overexpression of CNP in transgenic mice results in aberrant oligodendrocyte membrane expansion and inability of cytoplasmic membranes to fuse to form compact myelin (Gravel *et al.*, 1996). Myelin associated glycoprotein (MAG) is a quantitatively minor myelin protein (1% of myelin protein) composed of a transmembrane domain, an intracellular signalling domain and an extracellular, glycosylated immunoglobulin-like domain (MAG) (Figure 1B). MAG expression is dense near the nodes of Ranvier, where it is thought to contribute to the formation of the periaxonal cytoplasmic collar of myelin sheaths. Myelin oligodendrocyte glycoprotein (MOG) is a member of the immunoglobulin superfamily and a minor component of myelin. Despite the long-standing assumption that MOG was the only CNS specific myelin protein, MOG mRNA expression was recently demonstrated in peripheral myelin although protein expression was not detected (Pagany *et al.*, 2003). The MOG content of myelin was originally estimated to be 0.02% of CNS myelin, but a recent study calculated MOG to make up 2,5% of CNS white matter (Smith *et al.*,

2005). MOG is a transmembrane protein with an intracellular signalling domain and an extracellular domain that contains one glycosylation site (Figure 1B). MOG is expressed on the outermost lamellae of compact myelin. The physiological role of MOG is unknown. MOG knockout mice were phenotypically indistinguishable from wild type mice and no myelin changes were observed using light microscopy or electron microscopy (Delarasse *et al.*, 2003). Crosslinking of MOG by anti-MOG antibodies results in activation of proteins related to the cellular stress response and cytoskeletal stability (Marta *et al.*, 2005). The physiological ligand for MOG, and the relevance of MOG signalling in health and disease are unknown.

Other myelin(-associated) proteins include oligodendrocyte specific protein (OSP), myelin associated/oligodendrocyte basic protein (MOBP), oligodendrocyte-myelin glycoprotein (OMgp), myelin/oligodendrocyte specific protein (MOSP) and the small heat shock protein αB-crystallin (van Noort *et al.*, 2000; Baumann and Pham-Dinh, 2001).

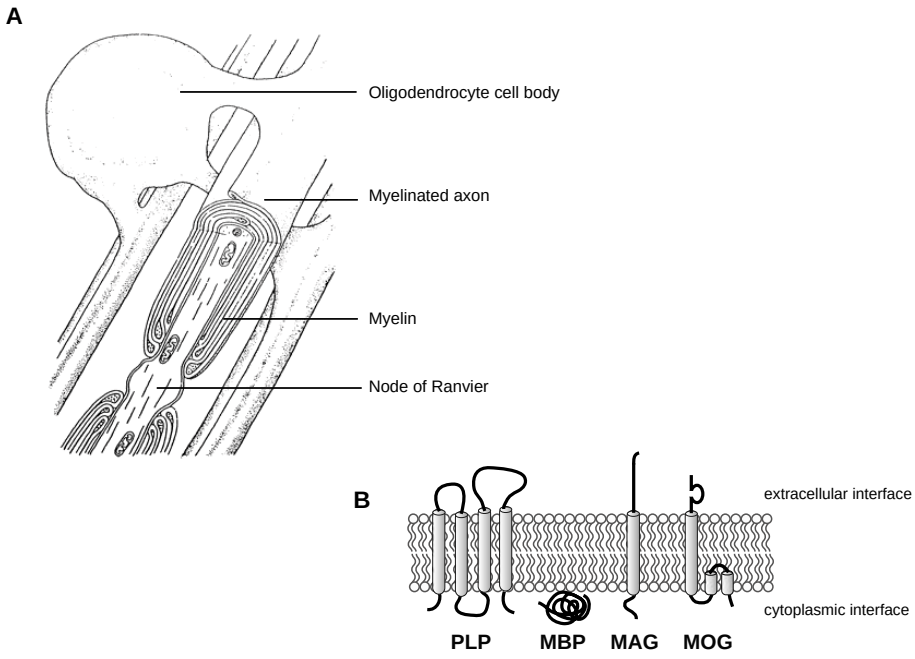


Figure 1.

Myelin and myelin proteins.

(A) Drawing of an oligodendrocyte and a myelinated axon shows wrapping of oligodendrocyte membranes into compact myelin (adapted from Bunge, 1968). (B) Schematic representation of CNS myelin proteins in the oligodendrocyte membrane.

1.3. Neuropathology

MS is classically considered as a disease of the CNS white matter, characterised by focal areas of myelin loss. However, a number of studies in the last decade have demonstrated that lesion formation and demyelination in grey matter areas are also common (Bö *et al.*, 2003b). MS lesions are most abundant in optic nerves, periventricular areas, brainstem, cerebellum, cerebral cortex and spinal cord (Lumsden, 1970). Whereas white matter lesions are characterized by infiltration of T cells and macrophages, leukocyte infiltration and inflammation are uncommon in grey matter lesions, suggesting that the factors contributing to MS lesion formation may be location-dependent (Peterson *et al.*, 2001; Bö *et al.*, 2003a; Brink *et al.*, 2005). Interestingly, axonal damage and axonal loss are common in both white and grey matter MS lesions, suggesting that both inflammatory and non-inflammatory mechanisms contribute to the neurological dysfunction in MS (Trapp *et al.*, 1998; Peterson *et al.*, 2001).
White matter lesions can be subdivided dependent on the inflammatory and demyelinating activity (van der Valk and de Groot, 2000). Inflammatory activity is characterized by the presence of macrophages/microglia expressing high levels of HLA-DR. Demyelinating activity is characterized by the presence of phagocytic macrophages/microglia containing intracellular myelin proteins (MBP, PLP or Luxol Fast Blue positive particles). Active demyelinating MS lesions show both inflammation and demyelination and are thought to represent ongoing demyelination (Lassmann *et al.*, 1998).

1.4. Neuroimmunology

Several paradigms have been proposed to explain the formation of MS lesions. Infiltration of the CNS parenchyma by activated T cells directed against myelin antigens, followed by accumulation of activated macrophages, may cause radially expanding lesions resulting in demyelinated plaques with an inflammatory border and astrogliosis in the centre (Hartung and Rieckmann, 1997). This hypothesis is based predominantly on results obtained in the animal model that is used to study MS *in vivo*, experimental allergic encephalomyelitis (EAE). Immunization of rodents or non-human primates with myelin antigens induces a peripheral T cell response directed against myelin. Activated T cells migrate to the CNS, cross the blood brain barrier (BBB) and enter the CNS parenchyma. The subsequent release of inflammatory mediators results in activation of microglia and recruitment of monocytes from the blood. Monocyte extravasation is an essential event for demyelination and clinical signs of EAE, as shown by the absence of clinical EAE in macrophage-depleted animals (Huitinga *et al.*, 1990; Huitinga *et al.*, 1995).
In contrast to EAE, in MS it is unknown what initiates the activation of T cells or even if activation of T cells is a primary event. Myelin specific T cells are present in the peripheral blood of MS patients but also in healthy donors (Sun *et al.*, 1991b; Lindert *et al.*, 1999; Andersson *et al.*, 2002). It is not exactly clear to what extent peripheral tolerance exists for myelin antigens. Not all myelin proteins are expressed in the thymus and in some cases such as MBP thymic expression is restricted to a different isoform, suggesting that some myelin antigens may be regarded as non-self (Klein *et al.*, 2000; Bruno *et al.*, 2002). Seclusion of myelin antigens from the periphery by the BBB has been suggested to prevent reactivation of myelin specific T cells by ignorance under normal circumstances. However, expression of

other myelin proteins was clearly demonstrated in the thymus and in non-immune peripheral tissue (Derbinski *et al.*, 2001; Bruno *et al.*, 2002), suggesting that the inactive state of myelin specific T cells in healthy individuals can not be explained by immunological ignorance. Recent studies suggest that the activation of myelin specific T cells in healthy individuals is actively suppressed by regulatory T cells (T_{reg}) or regulatory NK cells and that the functional activity of T_{reg} is reduced MS patients (Takahashi *et al.*, 2004; Viglietta *et al.*, 2004).

One theory for the activation of myelin specific T cells is by molecular mimicry after viral infection although superantigenic activation or bystander activation following microbial infection have also been proposed (Bronstein *et al.*, 1999a; Torres *et al.*, 2001; Haring *et al.*, 2002; Tejada-Simon *et al.*, 2003; Croxford *et al.*, 2005). However, most viruses that have been associated with MS are endemic in the normal population, suggesting that viral infection alone is not sufficient to induce MS (Stuve *et al.*, 2004).

Detailed analysis of active demyelinating MS lesions from patients with acute MS showed that four different immunopathological patterns of demyelination could be identified (Lucchinetti *et al.*, 2000). Pattern I lesions were compatible with the classical model of T cell initiated demyelination: lesions were centred around blood vessels and inflammatory infiltrates consisted of T cells and macrophages with a distribution suggestive of radial expansion. Macrophages and activated microglia were associated with degenerating myelin at the (well-defined) border of the lesions. Pattern II was characterized by depositions of complement and immunoglobulins at the site of active demyelination, in lesions that looked otherwise very similar to pattern I. Patterns III and IV were characterized by oligodendrocyte loss. Demyelination in pattern III lesions was not centred around blood vessels, and the border of the lesions was ill-defined. Oligodendrocytes in pattern III lesions showed preferential loss of MAG immunopositivity and were often apoptotic. Pattern IV lesions were distinct from pattern III as pattern IV lesions showed a well-defined border, with a rim of active demyelination and apoptotic oligodendrocytes. In pattern IV oligodendrocytes did not show preferential loss of MAG. The patterns of demyelination were heterogeneous between patients, but not within multiple active lesions from one patient. Thus, the heterogeneity of lesions may represent heterogeneity of pathogenesis (Lucchinetti *et al.*, 2000). Pattern II lesions were observed in 50-60% of patients, suggesting a role for antibody and complement mediated demyelination in a large proportion of MS patients. The patients included in this study all had acute MS with severe clinical episodes, it is unknown to what extent the heterogeneity of MS lesions is observed in patients with milder and chronic forms of MS.

Heterogeneity of MS lesions was not supported by a recent study, that suggested that oligodendrocyte apoptosis is the primary event in MS lesion formation, later followed by T cell infiltration and macrophage activation (Barnett and Prineas, 2004). Large areas of oligodendrocyte apoptosis were observed in acute MS lesions, the location of which could be linked directly to the fatal clinical event, demonstrating that these lesions were very recent. Infiltration of T cells and macrophages was absent in these apoptotic lesions, although mild activation of microglia and complement activation were observed. In the same patients, the more ‘classical’ MS lesions (patterns I and II), characterised by leukocyte infiltration were

observed as well. These lesions were suggested to represent a later stage of lesion formation, secondary to oligodendrocyte apoptosis.

Whether initiated by primary T cell activation or oligodendrocyte apoptosis, monocyte infiltration is thought to be an essential step in MS lesion formation. This was demonstrated in rodents depleted of blood monocytes, where clinical signs of EAE were suppressed (Huitinga *et al.*, 1990; Tran *et al.*, 1998). In addition, macrophages are the most abundant cell type in active demyelinating MS lesions, emphasizing the central role of macrophages in demyelination.

Macrophages are thought to contribute to CNS inflammation and demyelination by the release of inflammatory mediators and phagocytosis of myelin. Electron microscopy (EM) studies showed that macrophages in MS lesions are in direct contact with the axons (Prineas and Connell, 1978), and macrophage processes were observed to infiltrate between the axon and the myelin sheath, suggesting that macrophages directly 'eat' myelin from the axons. Macrophages express phagocytic receptors that directly bind myelin, such as scavenger receptors and lectin receptors (Mosley and Cuzner, 1996). In addition, macrophages express IgG receptors (Fcγ receptors) and complement receptors that bind myelin after opsonisation by antibodies and complement. Fcγ receptors and complement are discussed in more detail below.

Besides T cells and macrophages, a number of other cell types have been implicated in the formation of MS lesions, including mast cells, NK cells and B cells. Of these cells, the role of B cells in MS has been studied most extensively. A short overview of B cell development and antibody production is provided in Box 1.1.

Box 1.1. B cells and immunoglobulins

B cells originate from lymphoid precursors in the bone marrow and account for 10-15% of blood lymphocytes. Their main functions are production of immunoglobulins (Ig) and antigen presentation to T cells. Antigen (Ag) specificity of B cells is determined by the B cell receptor (BCR) that consists of a membrane bound Ig molecule associated with the Igα/Igβ heterodimer. Mature naïve B cells express low levels of low affinity membrane bound IgM molecules. Encounter of specific Ag induces B cell activation and differentiation, resulting in secretion of low affinity IgM in pentameric form. B cell activation occurs predominantly in the lymph nodes, where follicular dendritic cells, that have captured Ag in the form of immune complexes, present Ag to B cells. Like T cells, B cells require more than just the presence of specific Ag to become fully activated. The additional activating signal can be provided by $CD4^+$ T cells or by microbial Ag.

T cell-dependent B cell activation. After binding of protein Ag, B cells internalise the BCR-Ag complex, process the Ag and present it as a peptide in MHCII molecules. Recognition of the MHC-peptide complex by memory (but not naïve) $CD4^+$ T cells results in activation of B cells, but also in enhanced activation of the T cell through costimulatory interactions (such as CD40-CD40L and B7-CD28). B cells subsequently undergo somatic hypermutation of the IgV genes, resulting in the selection of high affinity BCR and production of high affinity antibodies (affinity maturation). In

addition, rearrangement of the heavy chain genes results in the selection of another IgFc tail, changing the antibody isotype from IgM to IgG, IgA or IgE (isotype switching). Isotype switching alters antibody effector function without altering Ag specificity. Differentiation and affinity maturation result in the generation of memory B cells that express a high affinity BCR, produce low levels of antibody and can undergo new cycles of activation and differentiation upon reencounter with the Ag. Alternatively, B cells may develop into end-differentiated plasma cells that have lost all surface Ig and continuously secrete high levels of antibody.

T cell-independent B cell activation. High doses of microbial Ag (for instance bacterial lipopolysacharide) activate B cells regardless of Ag specificity (polyclonal activation), whereas lower doses only activate Ag specific B cells although without T cell help. The alternative route of T cell-independent B cell activation is by repeating linear Ag that simultaneously crosslink multiple Ag specific BCR. Isotype switching or development of memory B cells do not occur after T cell independent B cell activation.

Antibodies and immune complexes. Immunoglobulins bind Ag in their native form, including conformational epitopes. This is in contrast to T cells that recognize peptide antigens (linear epitopes) in the context of MHC molecules. Binding of antibodies to soluble Ag results in the formation of immune complexes (IC), whereas binding to particulate Ag, expressed on the surface of microorganisms or cells, results in opsonisation of the particle, efficiently targeting the particles removal by mononuclear phagocytes. The effector functions of immunoglobulins depend on the Ig isotype. Binding of pentameric IgM to Ag results in efficient complement fixation, facilitating capture and removal of the Ag by phagocytes. IgG-containing IC are capable of complement fixation and crosslinking of leukocyte Fcγ receptors (FcγR), inducing a variety of effector functions (including FcγR mediated phagocytosis, Ag presentation and antibody dependent cytotoxicity). IgA antibodies are secreted as dimers in the lumina of mucosal surfaces and mainly act as neutralising antibodies, in blood IgA occurs as monomers with unknown function. IgE antibodies act as Ag receptors on mast cells by Fc-mediated binding to mast cell Fcε receptors that are capable of inducing potent inflammatory reactions after cross-linking by Ag (Peakman and Vergani, 1997; Janeway, Jr. *et al.*, 2001).

B cells as antigen presenting cells (APC). Although B cells are poor activators of naïve T cells, memory B cells are potent APC that were shown to be important for the propagation of T cell responses in several autoimmune diseases, including classically T cell mediated diseases (Falcone *et al.*, 1998; Takemura *et al.*, 2001).

2. B cells, antibodies, Fcγ receptors and complement in MS

B cells are observed in active demyelinating MS lesions and can contribute to lesion formation by the production of myelin specific antibodies (Baranzini *et al.*, 1999). A recent study described formation of ectopic B cell follicles in the meninges of MS patients, suggestive of intrathecal differentiation of B cells and local shaping of the antibody response (Serafini *et al.*, 2004). In addition, expression of CXCL13, a chemokine involved in chemoattraction of B cells and lymphoid neogenesis (Luther *et al.*, 2000; Cupedo and Mebius, 2003), in areas of perivascular infiltration suggests that similar processes may also occur at other locations in the MS brain (Corcione *et al.*, 2004). Furthermore, B cells can contribute to MS lesion formation from the periphery, by releasing antibodies directed against myelin antigens into the circulation. However, the role of B cells and the relevance of anti-myelin antibodies in MS is poorly understood, despite the overwhelming evidence for B cell activation in MS and the capacity of anti-myelin antibodies to exacerbate CNS inflammation and demyelination *in vivo* (Linington *et al.*, 1988).

2.1. CSF oligoclonal immunoglobulin bands and anti-myelin antibodies

CSF oligoclonal immunoglobulin bands (OCB) reflect intrathecal production of immunoglobulins by specific clones of B cells, directed against antigens that are present in the CNS. Using isoelectric focusing techniques, OCB are detected in at least ninety percent of MS patients (McLean *et al.*, 1990; Bourahoui *et al.*, 2004). The presence of OCB in the CSF of patients who present with clinically isolated syndrome (CIS) is predictive for the development of clinically definite MS (Paolino *et al.*, 1996). The absence of CSF OCB has been correlated with benign disease course, whereas high intrathecal immunoglobulin production has been associated with the most malignant forms of MS, suggesting that OCB may be clinically relevant (Correale and de los Milagros Bassani Molinas, 2002). However, OCB are also detected in a number of other neurological diseases, although not as consistently as in MS. In addition, OCB in other neurological diseases are usually transient (Correale and de los Milagros Bassani Molinas, 2002), whereas OCB in MS are persistent as shown by sequential analysis of CSF samples (Correale and de los Milagros Bassani Molinas, 2002; Bergamaschi *et al.*, 2004), suggesting ongoing intrathecal production of antibodies.

The antigen specificity of OCB in MS is largely unknown. It has been reported that OCB are detected against MBP and viral antigens, although generally the IgG in the OCB do not represent responses against myelin or infectious agents (Cross *et al.*, 2001; Correale and de los Milagros Bassani Molinas, 2002). In neurological diseases associated with the presence of foreign antigens in the CNS, the antigen specificity of OCB is usually directed against those foreign antigens, suggesting that OCB represent an antibody response driven by local antigens. It is possible that OCB in MS are directed against antigens that are yet unknown (p.e. viral antigens or modified self-antigens). OCB are not only detected in CSF, but can also be eluted from MS lesions (Warren and Catz, 1993), suggesting that at least part of the OCB found in the CSF are produced at the site of demyelination.

Apart from the presence of OCB, antibodies directed against a wide range of myelin antigens have been described in the CSF and serum of MS patients (Cross *et al.*, 2001; Correale and de los Milagros Bassani Molinas, 2002). The results obtained by the different laboratories are variable, probably due to the variety of antigens and methods that are used for screening although the differences most likely also reflect the heterogeneity of the anti-myelin antibody response in MS patients. Myelin specific antibody responses are not specific for MS, antibodies directed against myelin antigens are also detected in a number of other neurological diseases (such as viral and bacterial meningitis) and healthy donors (Reindl *et al.*, 1999). However, a subset of MS patients appears to show an enhanced anti-myelin antibody response. The available literature on anti-myelin antibodies in MS is extensive, therefore a selection of the literature is summarised below.

Generally, the presence of anti-myelin antibodies in CSF is more specific for MS than the presence of anti-myelin antibodies in serum. Enhanced CSF antibody responses against PLP, MBP, MOG, CNP, OSP and MAG were reported in MS patients when compared to healthy donors (Sun *et al.*, 1991b; Warren and Catz, 1994; Sellebjerg *et al.*, 1994; Walsh and Murray, 1998; Bronstein *et al.*, 1999b; Andersson *et al.*, 2002; Markovic *et al.*, 2003). In some studies the CSF anti-myelin antibody response correlated with active disease (Warren and Catz, 1989), but others could not confirm this (Markovic *et al.*, 2003). Although the CSF anti-MBP response has been reported to be specific for MS (Warren and Catz, 1999), most studies found production of anti-myelin antibodies in other inflammatory neurological diseases (OIND) as well (Sellebjerg *et al.*, 1994; Andersson *et al.*, 2002; Markovic *et al.*, 2003). Similar to the OCB the CSF IgG response to myelin antigens in MS seems to be persistent, whereas in OIND the response is usually transient (Link *et al.*, 1990; Sellebjerg *et al.*, 1994; Walsh and Murray, 1998).

Anti-myelin antibody responses in serum are more diverse and less specific for MS than those in the CSF. However, serum is easier to obtain than CSF, and the use of serum facilitates serial sampling. Therefore many groups have screened MS patients for serum antibodies directed against myelin proteins.

Most screenings for anti-myelin antibodies were performed in search of biomarkers to distinguish MS patients from healthy donors or to distinguish subgroups of MS patients, an approach that has recently shown promising results (Berger *et al.*, 2003). Elevated serum antibody responses or enhanced numbers of B cells secreting antibodies against MAG, MOG, PLP, αB-crystallin, MBP and CNP have been observed in MS patients when compared to healthy donors (Sun *et al.*, 1991a; Sun *et al.*, 1991b; Walsh and Murray, 1998; Lindert *et al.*, 1999; Schmidt *et al.*, 2001; Andersson *et al.*, 2002; Vojdani *et al.*, 2003; Gaertner *et al.*, 2004), whereas other studies reported that serum anti-myelin antibody responses in MS and healthy donors were comparable (Xiao *et al.*, 1991; Sellebjerg *et al.*, 1994; Lampasona *et al.*, 2004).

In summary, antibody responses against a wide variety of myelin proteins have been described in serum and CSF of MS patients, but none of these antibody specificities were specifically associated with MS. The most consistent finding was that enhanced anti-myelin antibody responses were restricted to a subpopulation of MS patients, supporting the hypothesis that

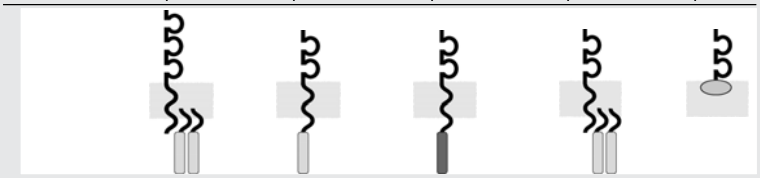
different pathogenetic mechanisms play a role in different groups of MS patients (Lucchinetti *et al.*, 2000). One study reported that the epitope specificity of anti-MOG antibodies in MS patients was different from healthy donors (Tejada-Simon *et al.*, 2002). This suggests that rather than being enhanced, anti-myelin antibody responses in MS may be directed against different epitopes. In addition, anti-myelin antibody responses may vary in different stages of the disease as a result of epitope spreading, as has been described for T cell epitopes in MS (Tuohy *et al.*, 1999). Less is known about determinant spreading of B cell epitopes, although the phenomenon has repeatedly been described in EAE (McFarland *et al.*, 1999; Bischof *et al.*, 2004). Another possibility is that it is necessary to combine the analysis of antibody responses against multiple myelin antigens to identify an anti-myelin antibody response specific for MS. As the combined studies performed thus far were limited to two or three myelin antigens, this remains to be addressed in the future.

Box 1.2. Fcγ receptors (FcγR)

Fcγ receptors (FcγR) efficiently combine humoral immunity with cellular immunity and innate immunity with adaptive immunity by enabling phagocytes to selectively phagocytose antibody-opsonised targets. In addition, co-expression of activating and inhibitory FcγR on myeloid and lymphoid cells provides a sensitive regulatory mechanism for activation of leukocytes (van de Winkel and Capel, 1993; Ravetch and Bolland, 2001).

Human FcγR The human FcγR system consists of three FcγR subclasses, FcγRI (CD64), FcγRII (CD32) and FcγRIII (CD16), all of which are encoded by genes on chromosome 1 (1q21-24). FcγR belong to the Ig superfamily and contain two (FcγRII and III) or three (FcγRI) Ig-like extracelullar domains, one transmembrane domain and cytoplasmic domains of variable length (van de Winkel and Capel, 1993) (table). FcγRIa (CD64) is a 72 kDa transmembrane protein and the only functional human FcγRI. FcγRIa is a high affinity receptor that is capable of binding monomeric IgG in addition to immune complexes (IC). For optimal surface expression and signal transduction, FcγRI depends on association with a dimer of the FcRγ chain that contains an intracellular immunoreceptor tyrosine-based activation motif (ITAM). In the absence of the FcRγ chain, surface expression of FcγRI is severely reduced and most effector functions are severely impaired. FcγRII (CD32) is a 40kDa transmembrane protein that binds IC. Human leukocytes express two functional FcγRII receptors, FcγRIIa and FcγRIIb. FcγRIIa is an activating receptor that contains an intracellular ITAM, and is independent of the FcRγ chain for functional expression and signalling. FcγRIIb is the only inhibitory FcγR found in humans and has an immunoreceptor tyrosine-based inhibitory motif (ITIM) in its intracellular domain. FcγRIII (CD16) is a 50-80 kDa protein, existing in two isoforms: FcγRIIIa and FcγRIIIb both of which bind IC. FcγRIIIa is a transmembrane receptor that is dependent on association with a signalling molecule for functional expression. FcγRIIIa primarily associates with a dimer of the FcRγ chain, but association with a dimer of the CD3ζ chain or a heterodimer of the γ and ζ chain is also possible (Letourneur *et al.*, 1991). FcγRIIIb is a glycophosphatidylinositol (GPI) linked molecule that lacks a transmembrane domain. FcγRIIIb is thought to capture IC without inducing inflammation (Daeron, 1997). The cellular expression of human FcγR is indicated in the table

Murine FcγR Several differences exist between the human and murine FcγR systems. Murine FcγRI is encoded on chromosome 3 whereas FcγRII and FcγRIII are encoded on chromosome 1. Murine FcγRI is a 70kDa transmembrane protein that depends on the FcRγ chain for signal transduction and surface expression, although residual expression was observed in absence of the FcRγ chain (Takai *et al.*, 1994; Barnes *et al.*, 2002). Murine FcγRI binds monomeric IgG2a and IC. Murine leukocytes express one FcγRII protein, the 40-60 kDa inhibitory FcγRIIb that signals through an intracellular ITIM and binds IC. Murine FcγRIIIa is a 40-60 kDa transmembrane protein that is fully dependent on a dimer the FcRγ chain for surface expression and signalling. FcγRIIIa binds IC. No other FcγRIII protein has been identified in mice. Cellular distribution of murine FcγR is indicated in the table.

Species	FcγRI	FcγRIIa[1]	FcγRIIb	FcγRIIIa[2]	FcγRIIIb[3]
					
Human	Macrophages, monocytes, neutrophils, eosinophils, DC, mast cells	Macrophages, monocytes, neutrophils, eosinophils, basophils, DC (subset), platelets, T cells (subset)	B cells, mast cells, basophils, macrophages, eosinophils, neutrophils, DC	Macrophages, monocytes, NK cells, eosinophils, DC, T cells (subset)	Neutrophil, eosinophils
IgG isotype specificity	3>1>4>>>2	R131: 3>1>>>2=4 H131: 3>1=2>>>4	3>1>4>>2	F158:1=3>>>2>>4 V158:1=3>>>2=4	1=3>>>2=4
Mouse	Monocytes, macrophages, DC	Not expressed	B cells , mast cells, macrophages, monocytes, neutrophils, DC, early thymocytes	Monocytes, macrophages, mast cells, neutrophils, NK cells, early thymocytes	Not expressed
IgG isotype specificity	2a>2b>1>>3		2b>1>>2a>>>3	1>2a>2b>>>3	

[1]A functional polymorphism in human FcγRIIa, expression of an arginine (R) or a histidine (H) at aminoacid position 131, defines affinity for IgG isotypes

[2]Human FcγRIIa is polymorphic due to expression of a valine (V) or a phenylalanine (F) at amino acid position 158.

[3]Human FcγRIIIb is expressed in two isoforms (NA1 and NA2) that share the same affinity for IgG isotypes but differentially affect internalisation of IC (see also Chapter 4).

2.2. Pathogenetic relevance of anti-myelin antibodies in MS

Although the reports on serum and CSF antibodies in MS are confusing, several lines of evidence suggest that antibodies actively contribute to MS pathogenesis. Evidence for the role of B cells in MS is mostly observational, however experimental studies have shown that antibodies directed against myelin components can contribute to inflammation and more importantly demyelination in the CNS. Studies on functional relevance of anti-myelin antibodies have focussed on antibodies directed against MOG, mostly because the pathogenic capacity of MOG and anti-MOG antibodies was unequivocally demonstrated in EAE (Linington *et al.*, 1988; Morris-Downes *et al.*, 2002). The functional role of B cells and antibodies in EAE is discussed in Chapter 2.

A recent study showed that serum anti-MOG antibodies in patients who present in the clinic with CIS, are a strong predictor for rapid progression to clinically definite MS. The association was even stronger when patients showed antibodies directed against both MOG and MBP (Berger *et al.*, 2003), suggesting that antibody responses against multiple myelin antigens contribute to disease progression. Pathological examination of active demyelinating MS lesions from a large group of early MS patients showed deposition of antibodies in fifty percent of patients (Lucchinetti *et al.*, 2000). Another study showed that part of the antibodies eluted from MS plaques were specific for MBP (Bernard *et al.*, 1981; Warren and Catz, 1993; Wucherpfennig *et al.*, 1997) and EM studies showed antibodies directed to MOG and MBP in association with degenerating myelin and within phagocytic macrophages (Genain *et al.*, 1999). This suggests that anti-myelin antibodies are associated with myelin phagocytosis in ongoing CNS demyelination. Antibodies can efficiently enhance myelin phagocytosis *in vitro* through crosslinking of Fcγ receptors (FcγR, Box 1.2), or by activation of the complement system (Box 1.3). Both FcγR and complement have been implicated in the formation of MS lesions.

2.3. Fcγ receptors in myelin phagocytosis and CNS inflammation

Microglia and perivascular macrophages, the resident macrophages of the CNS, constitutively express FcγRI, FcγRII and FcγRIII. In addition, low levels of FcγRIII are expressed on vascular endothelium in the CNS (Ulvestad *et al.*, 1994). In 1981, Prineas *et al.* demonstrated that macrophages located at the demyelinating edge of MS plaques showed polar capping with surface IgG, suggestive of receptor-mediated phagocytosis of antibody-opsonised particles (Prineas and Graham, 1981). This, in combination with enhanced expression of FcγRI and -II in MS lesions (Ulvestad *et al.*, 1994) suggests that FcγR- mediated phagocytosis contributes to demyelination. However, colocalisation of antibodies and FcγR in active demyelinating MS lesions is yet to be demonstrated.

In vitro studies showed that antibodies directed against myelin antigens enhanced uptake of myelin or myelin proteins by both macrophages and microglia in heat-inactived serum (Trotter *et al.*, 1986; Goldenberg *et al.*, 1989; Smith, 1993; Van der Goes *et al.*, 1999). Antibody-mediated uptake of myelin proteins was formally shown to be FcγR mediated by Abdul-Majid *et al.* (2002) who showed that antibody-mediated uptake of MOG was impaired in macrophages lacking functional expression of FcγRI and FcγRIII (Abdul-Majid *et al.*, 2002).

In addition to enhanced myelin phagocytosis, interactions between anti-myelin antibodies and FcγR can contribute to local inflammation in the CNS. The oxidative burst that is associated with myelin uptake by microglia was enhanced in presence of monoclonal anti-MBP IgG (Williams *et al.*, 1994). In addition, crosslinking of microglial FcγR by IgG-coated beads induced the production of superoxide and the release of inflammatory chemokines (Song *et al.*, 2002; Ueyama *et al.*, 2004). The role of antibodies and FcγR in CNS demyelinating disease was further addressed *in vivo* using different strains of B cell- and FcγR-knockout mice. These studies are discussed in Chapter 2.

2.4. Complement in myelin phagocytosis and CNS inflammation

Deposition of anti-myelin antibodies on myelin debris or directly on intact myelin sheaths may result in activation of the classical pathway of complement (Box1.3). Indeed, deposition of complement and IgG have often been observed within the same MS lesion (Gay *et al.*, 1997; Lucchinetti *et al.*, 2000; Barnett and Prineas, 2004), however colocalisation studies of complement and IgG on myelin sheaths, or in phagocytic cells, have not been performed. Analysis of myelin phagocytosis *in vitro* showed that antibody-mediated phagocytosis in fresh serum was significantly higher than in heat-inactivated serum, demonstrating cooperation of complement- and FcγR-mediated pathways (Van der Goes *et al.*, 1999).

Complement can also be activated by myelin in absence of antibodies (Vanguri *et al.*, 1982), through direct binding of C1q and C3 to myelin proteins (van der Laan *et al.*, 1996; Johns and Bernard, 1997). Antibody-independent complement activation can also occur through binding of C1q to apoptotic cells. This may have been the case in the acute MS lesions described by Barnett *et al.* (2004), where extensive oligodendrocyte apoptosis was accompanied by complement activation in absence of IgG.

Immunohistochemical studies on MS autopsy material demonstrated that active demyelinating MS lesions typically show a diffuse pattern of immunostaining for complement proteins and IgG, probably reflecting leakage of serum proteins through the locally disrupted blood brain barrier (BBB). In addition, complement activation products were observed in capillary walls, on astrocytes and occasionally on oligodendrocytes, myelin and neurons (Compston *et al.*, 1989; Gay and Esiri, 1991).

In areas of active demyelination, complement activation products and the terminal complex of complement activation (MAC) were observed on myelin sheaths or within phagocytic macrophages (Storch *et al.*, 1998; Barnett and Prineas, 2004). In fact, deposition of complement and IgG in active demyelinating lesions was suggested to represent a distinct immunopathological subtype of MS (Lucchinetti *et al.*, 2000).

Presence of complement activation products within phagocytic macrophages in active demyelinating MS lesions suggests a role for complement in myelin phagocytosis. Indeed, complement was shown to enhance myelin phagocytosis *in vitro* by opsonisation and uptake through complement receptor 3 (CR3), but also by MAC-mediated fragmentation of myelin particles facilitating myelin uptake by other receptors (van der Laan *et al.*, 1996; Mosley and Cuzner, 1996; DeJong and Smith, 1997). In addition, CR3 could mediate myelin phagocytosis in the absence of soluble complement, presumably through direct binding of CR3 to myelin

carbohydrates (Bruck and Friede, 1990a; Bruck and Friede, 1990b). Less is known on the role for other complement receptors in myelin phagocytosis. However, expression of CR4 and the phagocytic C1q receptor C1qRp on microglia and macrophages suggests that these receptors may contribute to myelin uptake as well (Webster *et al.*, 2000; Gasque *et al.*, 2000).
The origin of complement in MS lesions is unknown. The diffuse staining pattern in active demyelinating MS lesions is indicative of leakage from serum. However, resident brains cells produce low levels of complement proteins under normal physiological conditions (Shen *et al.*, 1997; Walker *et al.*, 1998; Thomas *et al.*, 2000; Hosokawa *et al.*, 2003), and expression of complement mRNA is upregulated during neuroinflammation, such as in Alzheimer's disease, Huntington's disease or bacterial meningitis (Veerhuis *et al.*, 1996; Stahel *et al.*, 1997; Yasojima *et al.*, 1999; Singhrao *et al.*, 1999). This, in addition to the expression of complement receptors in the CNS (Akiyama and McGeer, 1990; Gasque *et al.*, 1996; van Beek *et al.*, 2003) demonstrates that a local complement-mediated inflammatory response can be induced. It is unknown if local production of complement proteins plays a role in MS, although enhanced CSF complement protein concentrations in MS patients (corrected for leakage from peripheral blood) suggest intrathecal complement synthesis (Sellebjerg *et al.*, 1998).
The question of how complement activation affects lesion formation in MS remains. Whereas complement activation can be detrimental in acute CNS inflammation (e.g. cerebral ischaemia), complement activation and subsequent complement-mediated uptake of β-ameloid in Alzheimers' disease was suggested to be beneficial (van Beek *et al.*, 2003). Other regulatory effects of complement on CNS cells have also been described, such as complement-induced oligodendrocyte proliferation or complement-induced release of neural growth factors (Heese *et al.*, 1998; Soane *et al.*, 1999). *In vivo* studies on the role of complement activation in CNS demyelinating disease have yielded contradicting results and will be discussed in Chapter 2.

Box 1.3. The complement system

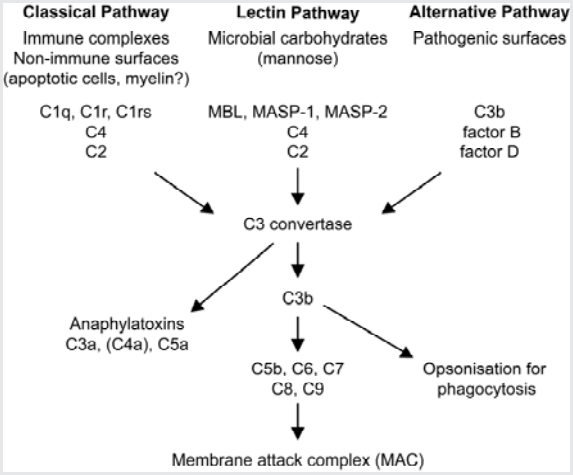
The complement system consists of a large group of plasma proteins that are inactive under normal physiological conditions. Activation of the complement system is initiated by binding of complement proteins to immune complexes (IC), pathogens or modified self-antigens. Binding of the first complement component induces a cascade of reactions that results in opsonisation or lysis of pathogens or clearance of apoptotic cells. Three pathways of complement activation have been identified, the classical pathway, the mannan-binding lectin (MBL) pathway and the alternative pathway (figure 1).

The classical pathway. The classical pathway is activated by binding of C1q to IC or to apoptotic cells. In its inactive state, C1q forms a complex with C1r and C1s. This complex disintegrates upon binding of C1q to IC, releasing C1r and C1s from the complex, thereby exposing an enzymatic site on C1r that cleaves C1s to become an active protease. C1s then initiates a cascade of reactions, leading to cleavage of complement C4, C2, C3 and C5. After cleavage one part of the protein is released as an inflammatory mediator (C2a, C4a, C3a and C5a) and the other part either acts as a new enzyme (C2b) to cleave other complement components or binds to the pathogenic or apoptotic

surface as an opsonin (C4b, C3b). Binding of C5b to C3b to the opsonised surface initiates the final pathway of complement that is shared by all three pathways. C5b forms a complex with C6, C7 and C8, which inserts itself into the membrane. C9 molecules then bind to this complex and polymerise, forming a pore in the pathogenic membrane - the membrane attack complex (MAC) - that contributes to lysis of the pathogen.

The MBL pathway. The MBL pathway is similar to the classical pathway. In its inactive state, MBL forms a complex with two inactive proteases, MASP-1 and MASP-2. Binding of MBL to carbohydrates on pathogenic surfaces releases and activates MASP-1 and MASP-2, resulting in cleavage of C4 and C2 and further activation of the complement cascade similarly to the classical pathway.

The alternative pathway. The alternative pathway is initiated by direct binding of C3 to pathogens, followed by activation of a series of proteins (factor B, factor D and factor P) that amplify the response by cleaving more C3 into C3a and C3b. C5b then binds to C3b, initiating the final pathway of complement.



Complement receptors. After complement opsonisation, pathogens or apoptotic cells can induce leukocyte activation or phagocytosis mediated by complement receptors (CR). CR1 (CD35) is expressed on myeloid cells and B cells and binds surface-bound C1q, C3b, C4b, MBL and the inactivated form of C3b (iC3b). CR1 cannot directly mediate phagocytosis, but the uptake of complement-opsonised targets under inflammatory conditions is greatly enhanced after CR1 crosslinking. B cells and follicular dendritic cells (FDC) recognize surface-bound C3d through CR2 (CD21). CR2 forms a complex with CD19, and crosslinking of this complex by C3d-opsonised antigens results in sustained B cell activation. CR3 (CD11b/CD18) and CR4 (CD11c/CD18) are expressed predominantly on myeloid cells and mediate phagocytosis of C3b opsonised targets.

3. Outline of the thesis

The role of humoral immunity in MS has received renewed attention in the last few years for a number of reasons (Lucchinetti *et al.*, 2000; Berger *et al.*, 2003). Anti-myelin antibodies are thought to contribute to MS pathogenesis in a subpopulation of patients, possibly through crosslinking of FcγR and complement activation, although the relative contribution of these effector pathways is unclear and require further study. It is, at present, not possible to identify MS patients with antibody-mediated pathology without further characterization using immunohistochemical methods on biopsy or autopsy CNS tissue. The availability of an easier and less invasive method to identify this subgroup of patients would provide a powerful tool to select patients who may benefit from immunotherapy aimed at antibody-mediated disease, such as intravenous immunoglobulins (IVIg). Development of a laboratory assay to identify anti-myelin antibodies in serum could possibly provide such a tool, provided that the test is able to reliably identify the pathogenically relevant antibodies.
The studies described in this thesis aimed to further elucidate the relevance of anti-myelin antibodies, FcγR- and complement-mediated mechanisms in MS and to identify antibodies directed against native human myelin antigens in the serum of MS patients.
In **Chapter 2** the role of humoral immunity in EAE is reviewed and in **Chapter 3** we address the role of FcγR in the induction of EAE and antibody-mediated demyelination. The relevance of genetic FcγR polymorphisms for MS susceptibility is addressed in **Chapter 4**. In **Chapter 5** we examined the extent of complement, antibody deposition and FcγR expression in active demyelinating lesions in chronic MS. **Chapter 6** describes a new assay to detect antibodies directed against whole myelin in serum of MS patients. The clinical relevance of serum anti-myelin antibodies is addressed in **Chapter 7**. In **Chapter 8**, the findings from this thesis are summarised in the context of recent developments in MS research.

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Chapter 2

B cells, antibodies, complement and Fcγ receptors in Experimental Allergic Encephalomyelitis (EAE)

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1. Experimental Allergic Encephalomyelitis (EAE)

A wide variety of models exist to study mechanisms underlying multiple sclerosis (MS) *in vivo*. CNS inflammation and demyelination can be induced in laboratory animals by immunisation with myelin antigens (experimental allergic encephalomyelitis, EAE), by infection with neurotropic viruses such as Theiler's murine encephalomyelitis virus (TMEV) or mouse hepatitis virus (MHV), or by intracerebral injection of toxins (e.g. cuprizone) (Van der Goes *et al.*, 2001). Immunological processes contributing to CNS inflammation and demyelination, including the role of humoral immunity, are mostly studied in models of EAE.

EAE can be induced in a number of rodent and primate species by immunisation with CNS homogenate or purified myelin proteins in adjuvant (active immunisation). Generally, immunisation with myelin antigens induces a T cell mediated autoimmune response. T cells specific for myelin antigens are activated in the periphery, migrate to the CNS, cross the blood brain barrier (BBB) and enter the CNS parenchyma. Local re-activation of myelin specific T cells results in the activation of microglia and perivascular macrophages and the recruitment of peripheral blood monocytes to the CNS parenchyma. Macrophages then start a local inflammatory and demyelinating response that results in the motor deficits that are the read-out system for clinical EAE.

The pivotal role of T cells has been demonstrated in models of adoptive transfer EAE (passive immunisation). Activated T cells from actively immunized animals can be isolated and transferred to naïve animals, resulting in a CNS inflammatory and demyelinating response and clinical signs of EAE (Trotter *et al.*, 1985).

However, the characteristics of EAE in the different models, such as disease incidence, day of disease onset, disease severity and the extent CNS inflammation and demyelination may depend on the additional factors, such as the activation of the humoral immune response. Antibodies and complement are thought to be particularly relevant for demyelination in chronic models of EAE. In addition, the development of antibody, FcγR and complement knockout mice in the last decade has provided some evidence that humoral mechanisms also contribute to the preclinical phase and the induction of EAE.

The contribution of the B cells, antibodies and complement is highly variable in the different models of EAE, and therefore it is often difficult to compare the results obtained in the different studies. In this chapter, we review the studies of humoral immunity in EAE and interpret the results, focussing on the role of B cells, antibodies, Fcγ receptors (FcγR) and complement in the induction and progression of EAE. Furthermore, we address the relevance of FcγR and complement in antibody-mediated demyelination.

2. Humoral immunity in EAE – the ‘ pre-knockout’ era

2.1. Antibody-mediated demyelination

Before the development of B cell deficient mouse strains it was technically difficult to dissect the role of T cells and B cells in the induction EAE. Therefore, the capacity of antibodies to contribute to CNS demyelination *in vivo* was addressed by injecting naïve animals with anti-myelin antibodies. The role of complement in EAE could be addressed directly by treating animals with cobra venom factor (CVF) resulting in rapid consumption and depletion of complement. Using a combination of these techniques, it was shown that sera of guinea pigs with spinal cord homogenate (SCH)-induced EAE could induce demyelination in naïve rats after intracerebral but not intravenous injection. Demyelinating activity was restricted to serum that was drawn from guinea pigs in the chronic phase of EAE, and the demyelinating capacity of EAE serum was abolished after complement depletion. Despite considerable demyelination, recipient rats did not show clinical signs of EAE (Lassmann *et al.*, 1981). These results highlight four characteristics of antibody-mediated demyelination that were repeatedly confirmed in later studies (Linington *et al.*, 1988; Morris-Downes *et al.*, 2002): (1) antibodies can induce demyelination *in vivo*, (2) antibodies can not induce clinical EAE in absence of a T cell response, (3) demyelinating antibodies are produced in the chronic phase of EAE, and (4) complement activation contributes to antibody-mediated demyelination.

Using the same approach, Linington and Lassmann showed in 1987 that the *in vivo* demyelinating capacity of guinea pig serum was correlated with the titre of anti-MOG antibodies, but not with anti-MBP and anti-PLP antibody titres (Linington and Lassmann, 1987). Anti-MOG antibody titres in guinea pigs with EAE increased over time, explaining that serum obtained in the chronic, but not the acute phase of EAE, could induce demyelination. Since then, studies on antibody-mediated demyelination have focused on anti-MOG antibodies.

Although unable to directly induce clinical EAE, anti-MOG antibodies have been shown to exacerbate clinical signs of EAE. A single injection with anti-MOG antibodies at the onset of acute MBP-induced EAE in rats resulted in exacerbation of clinical signs, associated with extensive plaque-like demyelination (Schluesener *et al.*, 1987; Linington *et al.*, 1988). This emphasises the demyelinating potential of anti-MOG antibodies, since demyelination in acute rat EAE is normally restricted to small perivenous areas. Similarly, anti-MOG antibodies could exacerbate MBP- or SCH-induced EAE in mice and MOG-induced EAE in marmosets (Schluesener *et al.*, 1987; Morris-Downes *et al.*, 2002; von Budingen *et al.*, 2004), whereas antibodies directed against MBP, PLP and GalC failed to induce a significant exacerbation of EAE (Morris-Downes *et al.*, 2002).

2.2. Complement in antibody mediated exacerbation of EAE

Injection of anti-MOG antibodies in animals with acute EAE results in massive activation of complement in areas of demyelination (Piddlesden *et al.*, 1993). In addition to diffuse complement staining in the CNS parenchyma, indicative of leakage of serum proteins through the BBB, complement C9 was observed in granular deposits on myelin sheaths and within phagocytic macrophages (Linington *et al.*, 1989a). The *in vitro* capacity of anti-MOG antibodies to fix

complement was directly related to their demyelinating potential *in vivo*, suggesting an essential role for complement activation in CNS demyelination (Piddlesden *et al.*, 1993). However, CVF treatment could not, or not completely, abolish anti-MOG antibody induced demyelination and leukocyte infiltration even though complement deposition was completely absent from the lesion (Linington *et al.*, 1989a; Piddlesden *et al.*, 1991). Nevertheless, the clinical severity of EAE was reduced in CVF treated animals, suggesting that complement contributes to exacerbation of clinical signs in antibody-exacerbated EAE (Linington *et al.*, 1989a). It is possible that the extensive complement activation following anti-MOG antibody injection contributes to EAE by direct induction of neuronal damage (Farkas *et al.*, 2003), but the extent of axonal damage was not addressed in the studies mentioned above.

CVF is a relatively crude method of complement depletion, associated with the transient release of inflammatory mediators that may influence the results. Therefore, complement depletion experiments were later repeated using a more sophisticated complement inhibitor, the soluble complement receptor 1 (sCR1). Rats treated with sCR1 before and during antibody-exacerbated EAE showed reduced clinical severity and reduced CNS inflammation and demyelination, confirming the relevance of complement activation for antibody-exacerbated disease (Piddlesden *et al.*, 1994). It is unknown if sCR1 completely blocked the effect of anti-MOG antibodies on EAE, as an EAE control group that was not treated with anti-MOG antibodies was not included in the study. However, the incomplete inhibition of antibody-exacerbated demyelination by CVF treatment suggests that in addition to complement, the other effector mechanism of antibody-mediated inflammation, crosslinking of FcγR, plays a role. This remains to be elucidated.

2.3. Antibody-independent complement activation in EAE

Complement depletion could prevent the induction of acute EAE in Lewis rats, in both the active immunisation model and the T-cell transfer model (Linington *et al.*, 1989b). Anti-myelin antibodies are virtually absent in models of acute rat EAE, suggesting that the contribution of complement to induction of acute EAE is antibody independent. Similar to antibody-enhanced complement activation, it is not exactly clear how direct complement activation contributes to clinical EAE, as CVF treatment did not prevent CNS inflammation in acute EAE. The suppressive effect of complement depletion on acute EAE could be overcome by transferring higher numbers of encephalitogenic T cell (Linington *et al.*, 1989b). Thus, complement activation is not essential for the induction of acute EAE in rats, but the presence of active complement lowers the threshold for developing clinical EAE.

In chronic EAE, complement deposition was most abundant during relapses in the chronic phase of disease (Linington *et al.*, 1989a). In this phase, anti-myelin antibodies are observed as well (Sadler *et al.*, 1991), probably explaining the enhanced complement activation in chronic EAE. Therefore, it is unknown if antibody-independent complement activation contributes to the chronic phase of EAE.

3. EAE in B cell-, FcγR- and complement deficient mice

With the development of mouse strains genetically deficient in specific components of the immune system, EAE research shifted from rats to mice. The role of B cells in EAE was addressed using several strains of μMT mice, that express a disrupted IgM μ-heavy chain, resulting in the complete absence of B cells (Kitamura *et al.*, 1991). Antibody-mediated effector functions were studied using mice lacking one or more FcγR and the role of complement was addressed using rodents lacking complement C3, C5, C6, factor B or complement regulatory proteins. The role of humoral immunity was studied almost exclusively in MOG-induced models of EAE, as MOG is the only myelin antigen that is alone sufficient to induce a CNS inflammatory demyelinating response that encompasses T- and B-cell responses (Adelmann *et al.*, 1995; Brok *et al.*, 2000).

3.1. Induction and progression of EAE in B cell deficient mice

The first studies of EAE in B cell deficient mice showed B cells were not essential for the induction of clinical signs of EAE. Upon immunisation with the MBP Ac1-11 peptide, B10.PL μMT mice (H-2^u) developed monophasic EAE with similar incidence, onset and severity as wt mice (Wolf *et al.*, 1996). Similarly, backcrosses of B10.PL and SJL/J mice (H-2^u/H-2^s) developed relapsing remitting EAE that was independent of B cells (Dittel *et al.*, 2000). In addition C57BL/6 μMT mice (H-2^b) were fully susceptible to induction and progression of chronic EAE after immunisation with recombinant mouse MOG (rmMOG) (Hjelmstrom *et al.*, 1998), with similar CNS inflammation and demyelination as wild type (Wt) mice. This suggests that B cells and antibodies are not essential for the induction, relapses or chronic phase of EAE and that demyelination can occur in the absence of antibodies. However, later studies showed that the picture is not that simple, and that B cells do contribute to EAE in other models.

In the case of MOG induced EAE, the relevance of B cells for the induction of EAE is dependent on the origin of the MOG protein. Recombinant MOG proteins derived from the rat or mouse MOG sequence (rrMOG and rmMOG) induced full-blown EAE in μMT C57BL/6 mice, whereas in the same mouse strain the induction of EAE with recombinant human MOG (rhMOG) was fully dependent on B cells (Lyons *et al.*, 1999; Oliver *et al.*, 2003). The difference was attributed to the poor immunogenicity of rhMOG. Due to a proline instead of a serine at amino acid position 42, rhMOG and MOG35-55 peptides derived from the human sequence induced a less efficient MOG specific T cell response than rMOG. Anti-MOG antibody production was comparable in response to rhMOG and rrMOG, but in the case of rhMOG immunisation, antibodies were critical for the induction of EAE as the T cell response alone was not sufficient (Oliver *et al.*, 2003). Transfer of serum anti-MOG antibodies to μMT mice (H-2^b) restored susceptibility to rhMOG EAE, with CNS inflammation and demyelination similar to Wt mice, even if serum antibodies were administered up to 31 days after immunisation (Lyons *et al.*, 2002). This demonstrates that B cells contribute to rhMOG EAE through production of anti-MOG antibodies, rather than through the APC function of B cells.

In three B cell deficient mouse strains of the H-2^q haplotype (μMT DBA/1, DBA/1-xid and μMT C57BL/10), rrMOG EAE was attenuated compared to Wt mice. The most prominent effect of B

cell deficiency was on demyelination but not inflammation, suggesting that B cells contribute to demyelination in H-2^q mice (Svensson *et al.*, 2002).

The capacity of mice to induce a demyelinating antibody response is related to the H2 haplotype of the mouse strain, which is thought to determine the capacity to induce antibodies directed against native conformational epitopes of the MOG protein. Upon immunisation with rMOG, H-2^s mice produced antibodies to the native MOG that were shown to be cytolytic *in vivo*. This was in contrast to H-2^b mice, that failed to produce antibodies directed against native MOG protein although production of antibodies to linear epitopes was normal (Bourquin *et al.*, 2003). However, although H-2^s are capable of producing demyelinating antibodies, the clinical severity of EAE in μMT mice of the H-2^s background was similar to Wt H-2^s mice (Dittel *et al.*, 2000). This does not exclude the possibility that B cell deficiency has an effect on demyelination, but this has not been evaluated.

The capacity of anti-MOG antibodies to contribute to an existing encephalitogenic T cell response was confirmed in a 'knock-in' mouse strains, genetically engineered to produce high levels of anti-MOG antibodies. Under normal physiological conditions anti-MOG antibodies were not pathogenic, but immunisation with rrMOG, PLP peptide or transfer of PLP specific T cells resulted in accelerated and more severe EAE associated with early demyelination (Litzenburger *et al.*, 1998). This demonstrates EAE can not only be exacerbated by injection of exogenous anti-MOG antibodies (Schluesener *et al.*, 1987; Linington *et al.*, 1988) but also by anti-MOG antibodies that are produced endogenously.

3.2. EAE in FcγR knockout mice

The balance between activating and inhibitory Fcγ receptors was shown to be very important in antibody-mediated autoimmune disease. FcRγ chain knockout mice (FcRγ$^{-/-}$ mice), lacking the activating FcγRI and FcγRIII (Takai *et al.*, 1994), were protected from collagen- and immune complex-induced arthritis (Ioan-Facsinay *et al.*, 2002), glomerulonephritis (Tarzi *et al.*, 2003) and vasculitis (Watanabe *et al.*, 1999). In contrast, mice deficient in the inhibitory FcγRII developed spontaneous glomerulonephritis (Bolland and Ravetch, 2000) and were more sensitive to collagen-induced arthritis (Kleinau *et al.*, 2000).

FcγR receptors have also been implicated in EAE. B6129PF2 FcRγ$^{-/-}$ mice did not develop clinical signs of EAE after immunisation with MOG35-55 (Lock *et al.*, 2002), and clinical signs were attenuated in FcγRIII$^{-/-}$ mice (Pedotti *et al.*, 2003), suggesting that both activating receptors contribute to the induction of EAE. In addition, DBA/1 FcRγ$^{-/-}$ mice were resistant to EAE after immunisation with rrMOG whereas EAE was more severe in FcγRII$^{-/-}$ mice (Abdul-Majid *et al.*, 2002). These results are somewhat surprising, as induction of EAE in all these models was independent of B cells (Lyons *et al.*, 1999; Svensson *et al.*, 2002). Although B cell deficiency reduced demyelination in DBA/1 mice, it appears unlikely that the complete absence of EAE in DBA/1 FcRγ$^{-/-}$ mice is related to the absence of IgG-FcγR interactions. In addition, significant anti-myelin antibody responses are usually not detected until the chronic phase of EAE (Morris *et al.*, 1997; Morris-Downes *et al.*, 2002; Pedotti *et al.*, 2003), suggesting that the absence of antibody-mediated effector functions would affect the chronic rather than the induction phase of EAE.

It is possible that IgG unrelated effector functions of the FcRγ chain contribute to the preclinical phase of EAE. The FcRγ chain also associates with the T cell receptor of γδT cells (Qian *et al.*, 1993), although the role of FcγR chain is unclear because FcRγ$^{-/-}$ mice do not show apparent abnormalities in the development of γδT cells (Takai *et al.*, 1994; Shores *et al.*, 1998). A functional role for γδT cells has been demonstrated in MOG35-55 induced EAE (Rajan *et al.*, 1996; Spahn *et al.*, 1999), suggesting that aberrant priming of γδT cells in FcRγ$^{-/-}$ mice may contribute to EAE resistance. The FcRγ chain has been shown to associate with at least five other receptor complexes on different cell types: (i) NKR-P1 on NK-, NK T - and dendritic cells (Arase *et al.*, 1997); (ii) αβTCR on NK T cells (Koyasu, 1994); (iii) PIR-A on dendritic cells, macrophages and B cells (Takai and Ono, 2001); (iv) GPIb and (v) GPIV on platelets (Wu *et al.*, 2001). Again, the role of the FcRγ chain in these complexes is ill defined. As a consequence the FcRγ$^{-/-}$ mouse has been considered almost exclusively as a mouse lacking activating FcγR, thereby possibly ignoring other deficiencies that may be relevant in autoimmunity.

During a CNS inflammatory response, microglia and macrophages show enhanced expression of FcγR (Ulvestad *et al.*, 1994). This, in addition to the fact that IgG are found within phagocytic macrophages in demyelinating lesions (Gay *et al.*, 1997), and the capacity of FcγR to enhance myelin phagocytosis *in vitro*, raises the question to what extent FcγR expression on macrophages contributes to the chronic phase of EAE (where considerable anti-myelin antibody levels can be measured). However, the effect of selective deletion of macrophage FcγR has not been studied in EAE.

In addition to FcγRI and FcγRIII, FcRγ$^{-/-}$ mice also lack expression of FcεRI, the high affinity receptor for IgE that is exclusively expressed on mast cells. Mast cells are implicated in the induction and progression of MOG35-55 induced EAE, as demonstrated by delayed onset, lower incidence and reduced severity of EAE in mast cell deficient mice (WBB6/F$_1$-Kit^{W}/Kit^{Wv}, H-2^{bxj}) (Secor *et al.*, 2000). Reconstitution of W/W^{v} mice with mast cells fully restored susceptibility to EAE, demonstrating that attenuated EAE was directly related to mast cell deficiency (Robbie-Ryan *et al.*, 2003). Interestingly, reconstitution with FcRγ$^{-/-}$ deficient mast cells could not restore EAE in W/W^{v} mice, implying a role for mast cell FcγR in EAE. Reconstitution experiments with mast cells lacking FcγRIII and FcγRIIb suggested an important role of IgG-FcγR interactions in the regulation of mast cell function in EAE (Robbie-Ryan *et al.*, 2003). The role of IgE and FcεRI remains to be established. Again, the role of mast cell FcγR in EAE is somewhat surprising, as MOG35-55 EAE was shown to be independent of B cells. It is possible that the encephalitogenic T cell response after MOG35-55 immunisation in wild type mice of the same genetic background (WBB6/F1-Kit^{+}/Kit^{+}) is somewhat milder than in C57BL/6 mice, explaining the requirement for additional factors to induce full-blown EAE.

3.3. EAE in complement deficient rodents

Whereas complement depletion with CVF could abolish clinical signs of EAE (Linington *et al.*, 1989b), deletion of individual complement components could never completely prevent the induction of EAE. In fact, studies in complement deficient rodents suggest that complement activation in the effector phase of EAE is at least as important as in the induction phase. The onset of MOG35-55 EAE in C57BL/6 mice deficient for C3 (C57BL/6 C3$^{-/-}$) and factor B (C57BL/

6 factor $B^{-/-}$) was similar as in Wt mice. However, disease severity was reduced in both knockout strains, presumably as a result of reduced CNS inflammation (Nataf *et al.*, 2000). Antigen specific proliferation of encephalitogenic T cells in $C3^{-/-}$ and factor $B^{-/-}$ mice was similar as in Wt mice, indicating that complement deficiency did not impair the T cell response after MOG35-55 immunisation (Nataf *et al.*, 2000).

In the alternative pathway of complement, cleavage products of C3 and FB amplify complement activation by forming a C3 convertase. Thus, $C3^{-/-}$ and factor $B^{-/-}$ mice lack the capacity to form the MAC, but also to generate large amounts of the inflammatory mediator C3a. This could explain attenuation of EAE, since C3aR knockout mice also showed reduced severity in the chronic phase of MOG35-55 EAE, associated with lower number of inflammatory cells in the CNS (Boos *et al.*, 2004). This was directly linked to the absence of C3a-C3aR interactions: CNS-restricted overexpression of C3a enhanced cellular infiltration and clinical signs, an effect that was abolished after deletion of C3aR (Boos *et al.*, 2004). The role of C3 in MOG35-55 EAE was not confirmed in another study (Calida *et al.*, 2001). In this study, a high dose of MOG35-55 and adjuvant were used to induce EAE, indicating that similar to the role of B cells in EAE, the additive role of complement can be overruled by an aggressive induction protocol yielding a more extensive T cell response.

Complement C5 appears to play different roles in the acute and chronic phases of EAE. Although clinical signs in the acute phase of EAE were comparable in C5 deficient and control mice, inflammation and particularly demyelination were reduced in C5 deficient mice. In contrast, in the chronic phase of EAE C5 deficient mice showed extensive axonal loss and astrogliotic scarring in the absence of remyelination, whereas C5 sufficient control mice showed large areas of remyelination, mild astrogliosis and limited axonal damage (Weerth *et al.*, 2003). Sublytic deposition of the membrane attack complex (MAC) on oligodendrocytes has been shown to protect oligodendrocytes against apoptosis and to induce proliferation (Rus *et al.*, 1997; Soane *et al.*, 1999). Indeed, oligodendrocyte apoptosis in C5 sufficient control mice was much lower than in C5 deficient mice, suggesting that C5 contributes to remyelination by MAC-mediated rescue and activation of oligodendrocytes (Niculescu *et al.*, 2004). This is further supported by the fact that EAE in $C5aR^{-/-}$ mice was not different from Wt mice, suggesting that the absence of C5b, rather than C5a, causes the EAE phenotype in C5 deficient mice (Reiman *et al.*, 2002). The regulatory function of MAC on remyelination and scar formation in chronic EAE remains to be confirmed in other studies.

The role of the membrane attack complex in acute MBP-EAE was further addressed in C6 deficient rats. In contrast to C3 and C5, C6 is not cleaved after complement activation and its function is restricted to the formation of the MAC. Thus, results obtained in C6 deficient animals are directly related to the inability to form the MAC. One study showed significantly reduced incidence, clinical severity, CNS inflammation and disease duration but normal T cell responses in C6 deficient rats with EAE (Tran *et al.*, 2002), but this was not confirmed in another study (Mead *et al.*, 2002). C6 deficiency protected against anti-MOG antibody mediated exacerbation of demyelination and axonal damage, confirming results obtained with sCR1 in antibody-exacerbated EAE (Piddlesden *et al.*, 1994; Mead *et al.*, 2002). Since all C6 deficient

rats were sacrificed within days after antibody injection, it is unclear how C6 deficiency affects remyelination and axonal damage in antibody-exacerbated EAE.

Complement activation in EAE has also been addressed at the level of complement regulators. Complement receptor related protein y (Crry) is a murine complement inhibitor that blocks the activation of multiple pathways of complement activation (Kim *et al.*, 1995). Transgenic C57BL/6xSJL/J-F1 (H-2^b or H-2^s background) mice that overexpressed soluble Crry in the CNS were resistant to MOG peptide induced EAE (H-2^s haplotype) or showed delayed onset (H-2^b haplotype) (Davoust *et al.*, 1999). In this study, Wt H-2^s mice displayed milder clinical signs of EAE than Wt H-2^b mice, again suggesting that complement activation may have an additive effect in mild clinical forms of EAE, which is redundant in more severe models. The capacity of soluble Crry to suppress or delay clinical EAE suggests a role for complement activation in the initiation of the encephalitogenic response, probably by reduced chemoattraction in the absence of C3a and C5a (Davoust *et al.*, 1999). CD59 is an ubiquitously expressed cell surface complement regulatory protein that protects self cells from MAC-induced lysis. Whereas Wt C57BL/6x129/Sv mice showed a mild form of EAE after immunisation with rrMOG, $CD59^{-/-}$ mice showed severe EAE. As C57BL/6x129/Sv fail to produce antibodies after rMOG immunisation, complement activation in $CD59^{-/-}$ mice is unrelated to antibody deposition (Mead *et al.*, 2004), demonstrating that antibody independent activation of MAC contributes to cellular infiltration, demyelination and axonal loss in EAE.

4. Summary and discussion

Generally, B cells, antibodies and complement are not essential for the induction of clinical EAE, although in some cases humoral immunity contributes to disease severity. In some models, EAE resistance in mice deficient for B cells or complement components could be overcome by using a more aggressive immunisation protocol, yielding a more efficient encephalitogenic T cell response. This suggests that humoral factors may lower the immunological threshold for the induction of clinical EAE if the encephalitogenic T cell response is weak.

It is not exactly clear how humoral factors contribute to the preclinical phase of EAE. The role of B cells in induction of EAE was related to production of anti-MOG antibodies rather than other B cell functions (Lyons *et al.*, 2002). Anti-myelin antibodies may contribute to local CNS inflammation by enhancing complement activation or cross-linking of FcγR resulting in amplification of the immune response, thereby facilitating the induction of clinical signs. However, the role of IgG-FcγR interactions in the induction of EAE is unclear, since the effect of FcγR deletion on EAE is much more dramatic than could be expected through the loss of IgG-FcγR interactions alone.

Complement proteins can directly enter the CNS parenchyma after disruption of the blood brain barrier, which precedes the onset of clinical EAE (Tonra *et al.*, 2001). Direct activation of complement by myelin antigens may induce a local inflammatory response that facilitates the induction of EAE by encephalitogenic T cells. Complement activation has also been implicated

in priming of T cells (Carroll, 2004), although this is less likely to play a role in the induction of EAE as T cell proliferation in complement deficient mice with attenuated EAE was normal (Nataf *et al.*, 2000).

The capacity of anti-myelin antibodies to contribute to ongoing CNS inflammation and demyelination was unequivocally demonstrated in models of antibody exacerbated EAE. Demyelinating capacity was restricted to anti-MOG antibodies. The role of endogenous anti-MOG antibodies in the effector phase of EAE is less clear. Although guinea pig and rat models showed a clear relation between anti-MOG antibody responses and demyelination, this was less evident in mouse models of EAE of demyelination in chronic EAE was in some models comparable in presence and absence of B cells.

The role of complement activation on the effector phase of EAE is even more complicated. Whereas soluble components of the complement cascade may have disease-promoting capacities early in the effector phase of EAE, the MAC may have a regulatory function in the late chronic phase of EAE.

An interesting thought is the possible redundancy of the complement and antibody-mediated inflammatory mechanims. Although it is unlikely that either mechanism is crucial for induction of EAE, it is possible that complement and antibody mediated inflammation are redundant in the effector phase of the disease and that mice deficient in both complement and B cells show differences in the chronic phase of disease. This remains to be addressed in the future.

In conclusion, humoral immunity may contribute to both the induction of and progression of EAE, although neither B cells nor complement are essential for clinical EAE in the presence of a strong encephalitogenic T cell response.

The capacity of B cells and complement may be very relevant in the pathogenesis of MS, where the initial T cell response may not be as potent as in EAE. Encephalitogenic T cells are found in the blood and CSF of MS patients, but also in patients with other neurological diseases and healthy donors (Lindert *et al.*, 1999; Andersson *et al.*, 2002), suggesting that additional factors are required to induce CNS demyelinating disease. Evidence from the *in vivo* models discussed in this review suggests that complement and myelin specific B cell responses can provide additional inflammatory signals that facilitate the induction of CNS inflammation and demyelination if the encephalitogenic T cell response is weak.

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Chapter 3

The FcRγ chain is not essential for induction of experimental allergic encephalomyelitis (EAE) or anti-myelin antibody mediated exacerbation of EAE

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Abstract

Macrophages are considered essential mediators in Multiple Sclerosis (MS) pathogenesis, presumably through myelin phagocytosis and release of inflammatory mediators. Macrophages and microglia express activating Fcγ receptors (FcγRI and FcγRIII), which depend on the FcRγ chain for surface expression and signalling. In MS lesions, crosslinking of FcγR by immunoglobulins (IgG) directed against myelin may enhance myelin phagocytosis and inflammation.

We studied the role of FcγR and anti-myelin antibodies in MOG35-55 induced experimental allergic encephalomyelitis (EAE) in C57BL/6 mice, a model of MS-like disease. Incidence and severity of EAE were similar in FcRγ chain$^{-/-}$ (FcRγ$^{-/-}$) and wild type (Wt) mice, albeit with delayed onset in FcRγ$^{-/-}$ mice. This demonstrates that the FcRγ chain is not essential for induction of EAE, but that FcRγ signaling may contribute to the preclinical phase.

The role of FcγR in antibody-mediated demyelination was addressed by injection of anti-myelin antibodies (Z12 mAb) at onset of MOG35-55 induced EAE. Injection of Z12 mAb rapidly reduced survival time, in both Wt and FcRγ$^{-/-}$ mice, demonstrating that antibody mediated exacerbation of EAE is independent of the FcRγ chain. Interestingly, Z12-induced exacerbation of inflammation and demyelination persisted longer in Wt than FcRγ$^{-/-}$ mice, suggesting that IgG-FcγR interactions may contribute to a sustained pathological effect of anti-myelin antibodies in the CNS.

Introduction

Multiple sclerosis (MS) is a chronic degenerative disease of the central nervous system (CNS), with poorly understood aetiology and pathogenesis. In experimental allergic encephalomyelitis (EAE), an animal model that is used to study (auto-) immune inflammation and demyelination in the CNS, both $CD4^+$ T and $CD8^+$ T cells specific for myelin antigens induce lesions highly reminiscent of MS lesions (Gold *et al.*, 2000). Importantly, T cells critically depend on macrophages to initiate demyelinating disease (Tran *et al.*, 1998). Abundant presence of activated microglia and macrophages containing intracellular myelin debris in active MS lesions, suggests that macrophages contribute to the formation of lesions by myelin phagocytosis. Several receptor families are implicated in myelin phagocytosis, including complement receptors and IgG receptors (FcγR) (van der Laan *et al.*, 1996; Van der Goes *et al.*, 1999; Reichert and Rotshenker, 2003). FcγR enhance phagocytosis of IgG-opsonised particles (Aderem and Underhill, 1999), thereby forming a bridge between adaptive and innate immunity. In MS lesions, expression of FcγR is enhanced on microglia and macrophages (Ulvestad *et al.*, 1994), and immunoglobulins are observed in a substantial proportion of active demyelinating MS lesions (Lucchinetti *et al.*, 2000). At least part of these immunoglobulins specifically bind myelin antigens, and can be found within phagocytic macrophages in association with degraded myelin (Genain *et al.*, 1999). However, it is unknown to what extent FcγR mediated myelin phagocytosis contributes to MS lesion formation.

The capacity of FcγR to contribute to myelin phagocytosis was demonstrated *in vitro*, where myelin specific antibodies enhanced myelin phagocytosis in absence of active complement (Van der Goes *et al.*, 1999). In addition, cultured microglia produced inflammatory chemokines upon FcγR crosslinking, further supporting a pathogenic role for IgG-FcγR interactions in the CNS (Song *et al.*, 2002).

Knockout mice lacking FcγR have improved the understanding of Ig-FcγR interactions in health and disease, including experimental autoimmune diseases (Hogarth, 2002; Takai, 2002). The murine leukocyte FcγR family consists of three subclasses (FcγRI, FcγRII, FcγRIII). The activating FcγRI and FcγRIII are expressed predominantly on myeloid cells and mediate inflammatory effector functions upon crosslinking by IgG-containing immune complexes (IC). For surface expression and signal transduction, both receptors depend on association with the FcRγ chain (Ravetch and Bolland, 2001). In FcRγ chain deficient mice ($FcR\gamma^{-/-}$), functional expression of FcγRI is severely impaired, while expression of FcγRIII is absent (Takai *et al.*, 1994; Barnes *et al.*, 2002). The inhibitory FcγRII is expressed on B cells and myeloid cells (Ravetch and Bolland, 2001). Crosslinking of FcγRII with activating FcγRI or FcγRIII results in down regulation of the activation signal. The balance between activating and inhibitory FcγR is critical for the regulation of antibody-mediated immune responses as demonstrated in several models of infectious and autoimmune diseases (Kleinau *et al.*, 2000; Ioan-Facsinay *et al.*, 2002; van Lent *et al.*, 2003; Nandakumar *et al.*, 2003).

Recent studies suggest an important role for both activating and inhibitory FcγR in the induction of EAE. Disease in $FcR\gamma^{-/-}$ mice was monophasic with low incidence and mild clinical symptoms (Lock *et al.*, 2002; Abdul-Majid *et al.*, 2002), while autoimmune prone $Fc\gamma RII^{-/-}$ mice exhibited

more pronounced disease (Abdul-Majid *et al.*, 2002). This led to the conclusion that interactions between anti-myelin antibodies and FcγR are important for the induction of EAE. It remains unclear if IgG-FcγR interactions can contribute to ongoing CNS demyelinating disease. Injection of antibodies directed against the immunodominant myelin oligodendrocyte glycoprotein (MOG) exacerbated EAE in rats and mice (Linington *et al.*, 1988; Morris-Downes *et al.*, 2002), implying that anti-MOG antibodies can contribute to ongoing CNS inflammation and demyelination. Antibody-mediated exacerbation of EAE was (partially) independent of complement (Piddlesden *et al.*, 1991; Morris-Downes *et al.*, 2002) suggesting involvement of Fc³R.

We explored the role of FcγR in the effector phase of EAE, using the MOG35-55 model that is B cell independent (Lyons *et al.*, 1999). Our hypothesis was that the FcRγ chain was not essential for the induction of MOG35-55 EAE, but that FcγR were instrumental in antibody-mediated exacerbation of EAE. Indeed, EAE was reproducibly induced in FcRγ$^{-/-}$ mice. However, injection of anti-MOG antibodies at onset of EAE rapidly exacerbated clinical EAE in both Wt and FcRγ$^{-/-}$ mice, demonstrating that anti-MOG antibodies can enhance clinical EAE independent of interactions with activating FcγR. Interestingly, sustained CNS inflammation and demyelination in Wt but not FcRγ$^{-/-}$ mice after injection of antibodies suggests that IgG-FcγR interactions may contribute to a sustained pathological effect of anti-MOG antibodies.

Materials and Methods

Animals

C57BL/6 mice were purchased from Harlan Olac (Horst, the Netherlands). FcRγ chain knockout mice (FcRγ$^{-/-}$) were generated on the C57BL/6 background in the lab of Dr. T. Saito (Park *et al.*, 1998). All mice were 10-20 weeks, weighing 18-25 g, at the time of EAE induction. Mice were specific pathogen free and had access to chow and water *ad libitum*. All experiments were performed with approval of the relevant ethical committees.

Antibodies

The monoclonal antibody (mAb) directed against MOG, Z12 mAb, is a mouse IgG2a. The hybridoma and ascites fluid were kindly provided by Dr. Sarah Piddelsden (Piddlesden *et al.*, 1993) and mAb was grown either at Charing Cross Hospital, London (UK) or at the VUMC, Amsterdam (The Netherlands). Z12 mAb was purified from supernatant or ascitic fluid by affinity chromatography using a protein A sepharose FF column (Amersham, Roosendaal, the Netherlands). Z12 F(ab')$_2$ fragments were produced in the lab of Dr. Van de Winkel. Hybridomas producing rat-anti-mouse mAbs 6B2 (anti-B220), KT3.1 (anti-CD3), M1/70 (anti-MAC-1) and M5/114 (anti-HLA-DR) were purchased from American Type Culture Collection (Manassas, US) and grown in our laboratory. Supernatants were used to detect T cells, B cells, macrophages and MHC Class II expression respectively. The 2.4G2 antibody (Pharmingen, Alphen a/d/ Rijn, the Netherlands) was used to detect expression of FcγRII/III.

Induction of EAE and assessment of clinical disease

Animals were immunized subcutaneously (s.c.) with 200 μg MOG peptide residues 35-55 corresponding to the mouse MOG peptide sequence (M-E-V-G-W-Y-R-S-P-F-S-R-V-V-H-L-Y-R-N-G-K) (Ansynth, Roosendaal, the Netherlands) emulsified in complete Freund's adjuvant (Difco, Detroit, US) (1:1, total volume 200 ¼l) supplemented with 600 μg heat killed *Mycobacterium tuberculosis* (Difco, Detroit, US). After 24 h, animals were injected intraperitoneally (i.p.) with 400 ng pertussis toxin derived from *Bordatella pertussis* (Sigma, Zwijndrecht, the Netherlands) in 200 μl saline (NaCl).

Animal were weighed and scored daily for clinical signs of EAE. Clinical disease was graded as follows: 0 = no clinical signs, 1 = limp tail, 2 = unsteady gait, hind limb weakness, 3 = incomplete hind limb paralysis, 4 = complete hind limb paralysis, 5 = moribund (euthanised) or death. Animals exhibiting signs of a lesser severity than typically observed were scored as 0.5 less than the indicated grade (Morris-Downes *et al.*, 2002).

Mice were sacrificed at day 16 or day 35 using O_2/CO_2. Serum was collected and stored at –80 ºC. CNS tissue was collected and stored at –80 ºC for immunocytochemistry or fixed in formalin before embedding in paraffin wax for histology.

Administration of MOG Z12 monoclonal antibody (Z12 mAb)

EAE was induced in C57BL/6 Wt or FcRγ$^{-/-}$ mice and at the onset of clinical signs (limp tail, grade 0.5-1), animals were injected i.p. with 1 mg of Z12 mAb, 1 mg Z12 F(ab')$_2$ or an equal

volume of saline (NaCl). As the day of onset varied between animals, mice were not all treated on the same day. To limit the variation in day of disease onset (and thus day of antibody injection) between treatment groups, animals were injected alternatingly with NaCl, Z12 mAb or Z12 F(ab')$_2$. The first animal to develop EAE was injected with NaCl, the second with Z12 mAb, the third with Z12 F(ab')$_2$, the fourth with NaCl, etc. As a result the average day of disease onset was comparable for all treatment groups within one strain.
As a control, C57BL/6 Wt mice were immunized with CFA, MTB and pertussis toxin in absence of MOG35-55 peptide and at the time corresponding to onset of EAE in Wt mice (day 14), the animals were injected i.p. with 1 mg Z12 mAb.

Histology, immunohistochemistry and immunocytochemistry

Mice were sacrificed 35 days after immunisation, and Kluver-Barrera (luxol fast blue/cresyl violet) staining was performed on formalin-fixed sections of spinal cord and cerebellum to assess demyelination (Baker *et al.*, 1990). Semi-quantitative evaluation of demyelination was performed blindly and each section was evaluated three times. Perivascular demyelination, small rims of demyelination centred around blood vessels, and plaque like demyelination, larger areas of myelin loss that were not obviously associated with blood vessels, were scored independently as absent (-), minor (+), moderate (++), intermediate (+++) or extensive (++++). Total demyelination, the cumulative score for perivascular and plaque-like demyelination was used for statistical analysis.
Immunocytochemistry on frozen material was used to detect infiltration of macrophages, T cells, B cells, the extent of immune activation (estimated by MHC Class II expression) and expression of FcγRII and FcγRIII. CNS tissue sections (5 ¼m) were fixed in acetone, followed by incubation with phosphate buffered saline (PBS) containing 0.1 % bovine serum albumin (BSA; PAA laboratories, Linz, Austria) and 5% normal mouse serum to block non-specific and Fc-mediated interactions. After rinsing in PBS, sections were incubated with primary antibody in PBS/0.1% BSA (1 h, 20 ºC), followed by another rinse with PBS and incubation with peroxidase (HRP)-conjugated rabbit-anti-rat Ig secondary antibody (DAKO, Glostrup, Denmark) in PBS/0.1% BSA (1 h, 20 ºC). After rinsing with PBS, peroxidase activity was visualized using 0.5 mg/ml 3,3'-diaminobenzidine tetrahydrochloride (DAB) (Sigma, Zwijndrecht, the Netherlands) in 0.05 M Tris-HCl buffer (pH 7.6) and 0.03 % H_2O_2. Immunostaining was assessed quantitatively using the computer program AnalySis (Soft Imaging System GmbH, Münster, Germany), and expressed as the percentage of DAB-positive area in the cerebellum white matter relative to the total cerebellum white matter area. In each group, sections from at least five mice were analysed, unless stated otherwise.

Statistical analysis

Differences in day of disease onset, clinical score and macrophage infiltration between experimental groups were assessed using ANOVA and Student's t-test. Disease incidence and survival were analysed using Pearson's Chi-square test. The extent of demyelination in different experimental groups was compared using Mann-Whitney U-test. Kaplan-Meier analysis and log-rank tests were used to analyse differences in survival of Wt and FcRγ$^{-/-}$ mice after injection of Z12 mAb or saline.

Results

FcRγ$^{-/-}$ mice develop chronic EAE

EAE was induced in C57BL/6 Wt and FcRγ$^{-/-}$ mice using MOG 35-55 peptide. FcRγ$^{-/-}$ mice developed chronic disease with similar clinical characteristics as Wt mice, albeit with significantly delayed onset (day 15.8 ± 3.6 vs. day 9.4 ± 3.1 respectively; $p<0.05$) (figure 1A). No significant differences were observed between FcRγ$^{-/-}$ and Wt mice with respect to disease incidence (13/16 and 28/31 respectively), maximal clinical score (4.3 ± 0.5 and 4.0 ± 0.5 respectively) and survival (9/13 and 8/9 respectively) (table 1).

Histopathology of EAE in Wt and FcRγ$^{-/-}$ mice was evaluated at 35 days after immunisation. CNS macrophage infiltration, estimated by the mac-1 positive area in the cerebellar white matter, did not differ between Wt and FcRγ$^{-/-}$ mice (figure 1B, table 1). Kluver-Barrera staining showed that demyelination was limited and mostly perivascular in both strains (data not shown), with no significant differences between Wt and FcRγ$^{-/-}$ mice (table 1). The experiment was repeated three times, yielding similar results.

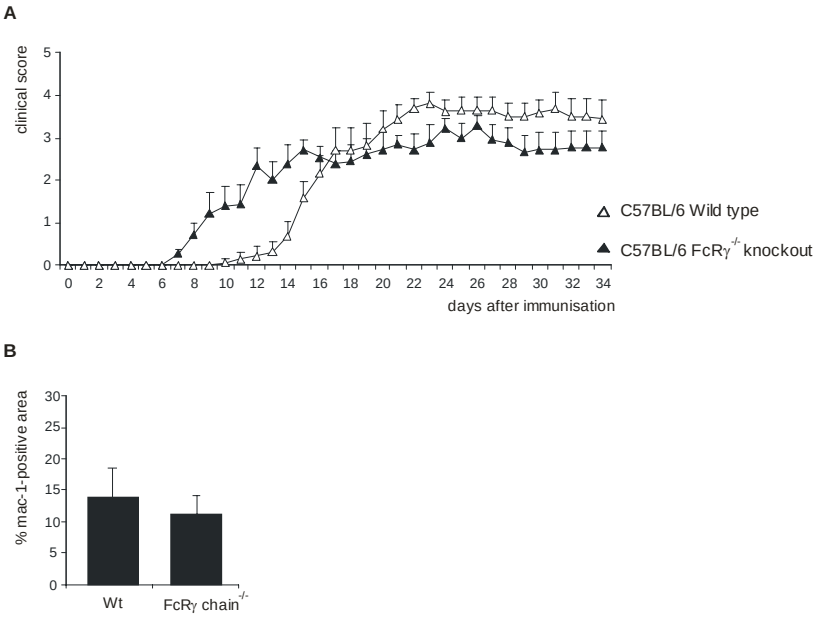

Figure 1.

Clinical course (A) and CNS macrophage infiltration (B) after induction of MOG35-55 EAE in Wt and FcRγ$^{-/-}$ C57BL/6 mice. A. Onset of EAE was significantly delayed in FcRγ$^{-/-}$ (open triangles, n=13) mice compared to Wt mice (filled triangles, n = 9) ($p<0.05$) although clinical course and severity of EAE were similar. The figure is representative of three independent experiments. (B) Quantitative analysis of macrophage infiltration in cerebellum white matter at 35 days after immunisation showed no significant differences between Wt and FcRγ$^{-/-}$ mice (n=7 for both strains). Macrophage infiltration was expressed as the percentage of mac-1 positive area compared to total cerebellum white matter area.

Table 1. Clinical characteristics of MOG35-55 EAE in C57BL/6 Wt and FcRγ$^{-/-}$ mice

Mice (n)	Incidence	Day of onset (±stdev)	EAE survival[c]	Maximal score (±stdev)	Demyelination[d]	Macrophage infiltration (±se)[e]
Wt (31)[a]	28/31	9.4 (3.1)	8/9	4.0 (0.5)	1.5	13.9 (4.4)
FcRγ$^{-/-}$ (16)	13/16	15.8 (3.6)[b]	9/13	4.3 (0.5)	2.0	11.3 (2.9)

[a] Of the 28 wt mice that developped clinical EAE, 9 mice were randomly selected to study clinical course of EAE, maximal clinical score, CNS demyelination and macrophage infiltration. The other Wt mice were included in other studies
[b] Significantly delayed compared to Wt mice ($p<0.05$, Student's t-test)
[c] Survival up to at least 35 days after immunization, only animals exhibiting clinical signs of EAE were included in the survival study
[d] Cumulative perivascular and plaquelike demyelination (median); day 35
[e] [mac-1 positive area in cerebellum white matter] / [total cerebellum white matter area] x 100; day 35

Exacerbation of EAE after injection of Z12 mAb is independent of the FcRγ chain

The capacity of IgG-FcγR interactions to contribute to ongoing CNS inflammation and demyelination was addressed using a model of antibody-augmented EAE (Linington *et al.*, 1988; Morris-Downes *et al.*, 2002).

We first investigated if Z12 mAb was able to exacerbate MOG35-55 induced EAE in C57BL/6 Wt mice. A single injection of Z12 mAb at onset of clinical EAE significantly enhanced clinical signs of EAE (figure 2; Z12 mAb injection vs. NaCl injection, $p<0.05$, day 1-4, day 9-12). The disease-modifying effect of Z12 mAb was dependent on the Fc portion of the antibody, as injection of 1 mg Z12 $F(ab')_2$ fragments did not exacerbate clinical EAE (figure 2).

The experiment was repeated using FcRγ$^{-/-}$ mice and a Wt control group. Injection of Z12 mAb dramatically enhanced clinical EAE in both Wt and FcRγ$^{-/-}$ mice. Survival of saline-injected mice after 35 days was 7/7 in both strains whereas survival after injection of Z12 mAb was significantly reduced in both Wt and FcRγ$^{-/-}$ mice (2/7 and 3/7 respectively; $p<0.05$ when compared with saline injected mice, Kaplan-Meier analysis and Log-rank test) (figure 3). C57BL/6 Wt mice that died after injection Z12 mAb succumbed at 2.8 ± 0.4 days after injection, FcRγ$^{-/-}$ mice succumbed significantly later at 4.0 ± 0.8 days ($p<0.05$). The effect of antibody injection on survival was short-lived; animals either died within 5 days after antibody injection or survived for the remainder of the experiment. The maximal clinical score in Wt mice was significantly higher after Z12 injection than after saline injection (4.6 ± 0.8 and 3.5 ± 0.8 respectively; $p<0.05$), no such difference was observed in FcRγ$^{-/-}$ mice (4.6 ± 0.5 and 4.3 ± 0.3 respectively). It must be noted that the maximal clinical score of Wt mice in this experiment was somewhat lower than in the previous experiment, whereas clinical scores of FcRγ$^{-/-}$ mice were similarly high (compare table 1 and table 2). As a result, an increase of clinical scores after injection of Z12 mAb is easier to measure in Wt mice than in FcRγ$^{-/-}$ mice, because in the latter a possible increase in clinical signs may be obscured by high clinical

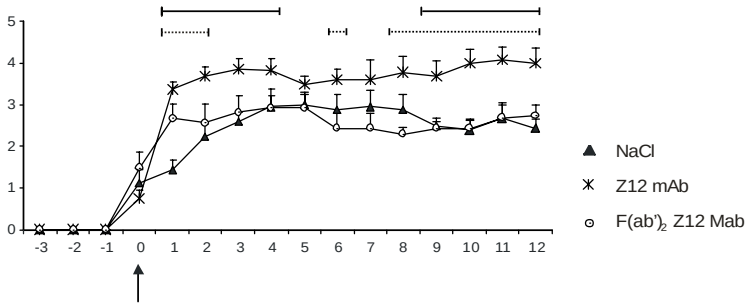

Figure 2.

Disease modifying effect of Z12 mAb onb MOG35-55 EAE in C57Bl/.6 mice. EAE was induced in C57BL.6 AWt mice and at first appearance of clinical signs (arrow) animals were injected with 1 mg Z12 mAb (crosses, n = 8), Z12 $F(ab)_2$ fragments (open circles, n = 8) or an equal volume of NaCl (filled triangles, n = 9). Injection of Z12 mAb, but not Z12 $F(ab')_2$ fragments resulted in exacerbation of clinical EAE. Figure is representative of three independent experiments (black lines: NaCl vs. Z12 mAb, Student's t-test $p<0.05$; dashed lines: Z12 $F'(ab)_2$ vs. Z12 mAb, Student's t-test $p<0.05$).

Table 2. Clinical characteristics of EAE in Wt and FcRγ$^{-/-}$ mice after injection of anti-MOG antibodies

Mice (n)	Treatment	Maximal score (±stdev)	EAE survival[c]	Day of onset (±stdev)	Demyelination[f]	Macrophage infiltration
Wt (7)	NaCl	3.5 (0.8)	7/7	14.5 (0.7)	2.0	6.6 (1.9)
Wt (7)	Z12	4.6 (0.8)[a]	2/7[d]	14.5 (0.7)	5.0[g]	15.0 (5.5)
FcRγ$^{-/-}$ (7)	NaCl	4.3 (0.3)[b]	7/7	16.9 (0.5)[e]	1.5	11.0 (4.4)
FcRγ$^{-/-}$ (7)	Z12	4.6 (0.5)[b]	3/7[d]	16.9 (0.5)[e]	1.5	8.3 (3.3)
Wt CFA-ctrl(4)	Z12	0.0 (0.0)	4/4	-	0	0.43 (0.25)

[a]Significantly higher than Wt NaCl-inected mice ($p<0.05$, Student's t-test)
[b]Significantly higher than Wt NaCl-inected mice ($p<0.05$, Student's t-test)
[c]Survival up to at least 35 days after immunisation, only animals exhibiting clinical signs of EAE were included in the survival study
[d]Significantly lower than NaCl-injected mice ($p<0.05$, Pearson Chi square)
[e]Significantly later than Wt mice ($p<0.05$, Kaplan-Meier analysis and Log Rank test)
[f]Cumulative perivascular and plaquelike demyelinatoin (median); day 35
[g]Significantly higher than Wt NaCl-injected mice ($p<0.05$, Mann-Whitney test)
[h] [mac-1-positive area in cerebellum white matter] / [total cerebellum white matter area] x 100, day 35

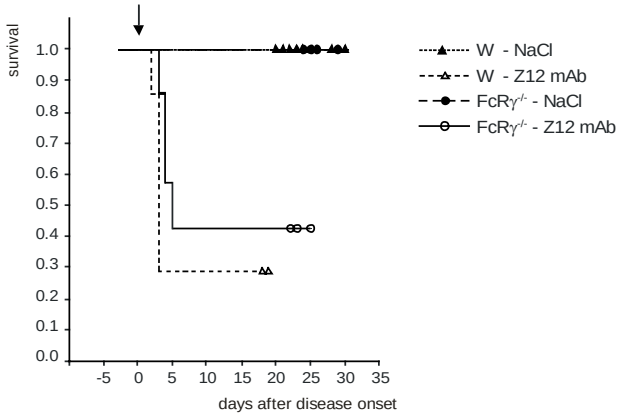

Figure 3.

Kaplan-Meier analysis of survival of C57BL/6 Wt and FcRγ$^{-/-}$ mice after injection of Z12 mAb at onset of MOG35-55 EAE. EAE was induced in Wt (triangles, n=7) and FcRγ$^{-/-}$ (circles, n=7) mice, and animals were injected with 1 mg of Z12 mAb (open symbols) or and equal volume of saline (filled symbols) at disease onset. Z12 mAb significantly reduced survival in both Wt and FcRγ$^{-/-}$ mice within the first five days after injection (*p<0.05, Log Rank test).

scores of EAE even in absence of antibody. Exacerbation of EAE after Z12 injection in Wt mice was observed in three independent experiments. This demonstrates that the effect of anti-MOG antibody injection is reproducible, even if there is variability in clinical scores between experiments, a well-known problem of EAE in C57BL/6 mice.

Injection of Z12 mAb in CFA control mice did not cause EAE or reduce survival, confirming that anti-MOG antibodies can exacerbate but not cause clinical EAE and that injection of Z12 in absence of an ongoing CNS inflammatory response is not lethal (table 2).

Sustained effect of Z12 mAb injection on demyelination in Wt but not in FcRγ$^{-/-}$ mice

Mice that survived injection of Z12 mAb and Z12 F(ab')$_2$- and saline-injected mice were sacrificed 35 days after immunisation and the CNS was isolated for histopathological examination. This was on average 21 days (range: 18-25 days) after injection of Z12 mAb, Z12 F(ab')$_2$ or saline. Spinal cords from Z12 mAb-injected mice, but not from NaCl-injected mice, were in such a poor condition presumably due to the severity of disease in the CNS that they could not be extracted. Thus we examined cellular infiltration and demyelination in the cerebellum, which is also a good and relevant measure of disease activity in chronic EAE (Zavala *et al.*, 2002; Nagelkerken *et al.*, 2004).

C57BL/6 Wt mice injected with Z12 mAb showed extensive plaque-like and perivascular demyelination, compared to limited perivascular demyelination in saline injected mice (figure 4A,B; table 2). The difference between saline and Z12 mAb injected Wt mice was significant ($p<0.05$). Unlike previous studies (Linington *et al.*, 1988; Piddlesden *et al.*, 1993; Morris-Downes *et al.*, 2002) we carried out this pathological examination of the brains approximately

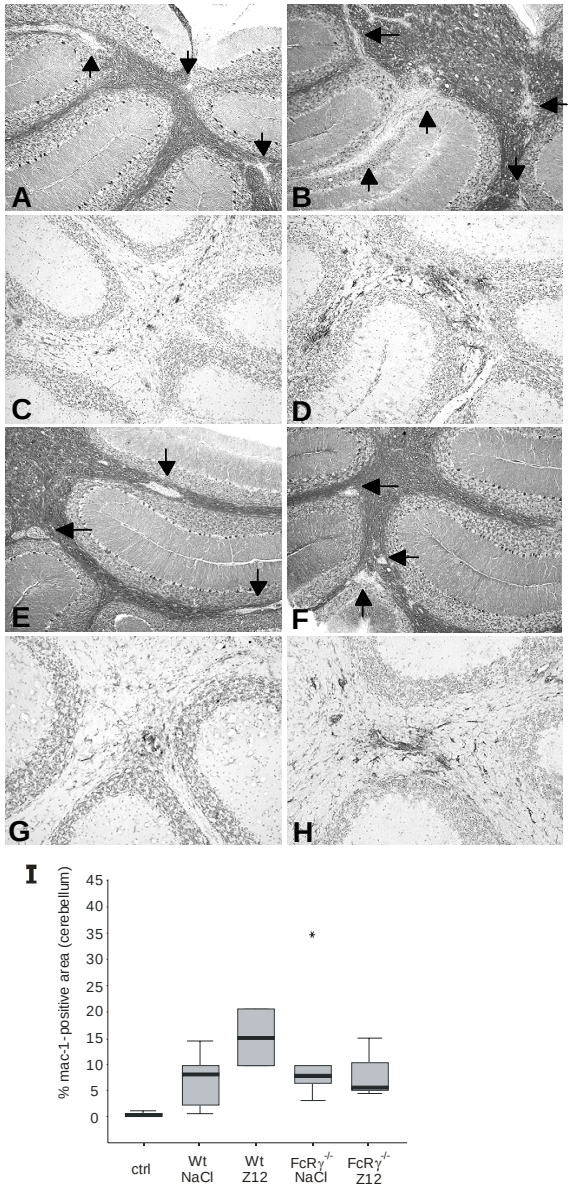

Figure 4.

CNS inflammation and demyelination in anti-MOG antibody exacerbated EAE in Wt and FcR$\gamma^{-/-}$ mice. Brains were isolated 35 days after immunisation, and the cerebellum white matter was analysed for demyelination using Kluver-Barrera staining (A,B,E,F) and inflammation using mac-1 staining (C,D,G,H). In Wt mice, injection of Z12 mAb resulted in enhanced demyelination (B) and inflammation (D) when compared to saline-injected animals (A and C respectively). In FcR$\gamma^{-/-}$ mice, injection of Z12 mAb did not change demyelination (F) or inflammation (H) compared to saline-treated mice (E,G). Areas of demyelination are indicated by arrows. Original magnification 40x. (I) Quantitative analysis of macrophage infiltration (percentages calculated as [mac-1-positive area/total cerebellum white matter area] x 100). Data represent average of 7 mice (Wt-NaCl; FcR$\gamma^{-/-}$-NaCl), 2 mice (Wt-Z12 mAb) or 3 mice (FcγR$^{-/-}$-Z12 mAb).

See page 162 for a full-colour representation of figure 4A-H.

3 weeks after injection of Z12 mAb, demonstrating that the effect of antibody administration was not limited to the first days after injection. In Wt mice that were injected with Z12 $F(ab')_2$ fragments, demyelination at day 35 was comparable to demyelination in Wt mice (data not shown), suggesting that the sustained effect of anti-MOG antibody injection is dependent on the Fc part of the antibody. Interestingly, FcRγ$^{-/-}$ mice did not show sustained CNS pathology after injection of Z12 mAb. Three weeks after injection of anti-MOG antibodies, demyelination in Z12 mAb treated FcRγ$^{-/-}$ mice did not differ from demyelination in saline treated FcRγ$^{-/-}$ or Wt mice (figure 4E,F; table 2). This suggests that IgG-FcγR interactions contribute to the sustained effect of Z12 mAb on demyelination.

Macrophages are thought to actively contribute to demyelination by myelin phagocytosis, therefore we subsequently evaluated macrophage infiltration in the CNS. In Wt mice, macrophage infiltration at day 35 was enhanced after injection of Z12 mAb (figure 4C,D; table 2). Quantitative analysis of mac-1 staining confirmed enhanced macrophage infiltration in Wt mice after injection of Z12 mAb (figure 4I). This difference did not reach statistical significance, probably as a result of the high variation within experimental groups and the low number of mice analysed in the Z12 mAb injected group. However, similar results were obtained in three independent EAE experiments. Macrophage infiltration in Z12 $F(ab')_2$ treated animals was comparable to saline treated animals (data not shown).

Although macrophage infiltration at day 35 in saline treated FcRγ$^{-/-}$ appeared to be somewhat higher than in saline treated Wt mice, this difference was not significant (table 2). A possible trend towards higher macrophage infiltration in FcRγ$^{-/-}$ mice may reflect the difference in clinical scores between Wt and FcRγ$^{-/-}$ mice in this experiment (figure 4C,G; table 2). Importantly, injection of Z12 mAb in FcRγ$^{-/-}$ mice did not enhance macrophage infiltration when compared to injection of saline (figure 4G,H,I). Again, this suggests that the FcRγ chain may contribute to the sustained inflammation and demyelination after injection of anti-MOG antibodies.

Discussion

This study demonstrates that induction and progression of MOG35-55 EAE are independent of the FcRγ chain, although delayed onset of EAE in FcRγ$^{-/-}$ mice suggests a role for FcRγ signalling in the preclinical phase of EAE. Furthermore, interactions between IgG and activating FcγR are not essential for anti-MOG antibody mediated exacerbation of EAE. However, IgG-FcγR interactions may contribute to a sustained effect of anti-MOG antibodies on CNS inflammation and demyelination.

The relevance of B cells and antibodies in EAE is debated, and the interpretation of data is complicated by the variety of models that are used. The role of B cells is best characterized in MOG-induced EAE in C57BL/6 and DBA/1 mice. Consequently, the role of FcγR has been addressed in these models (Lock *et al.*, 2002; Abdul-Majid *et al.*, 2002).

In C57BL/6 mice, the origin of the MOG protein is crucial for the role of B cells in EAE. Induction of EAE using recombinant human MOG (rhMOG) is B cell dependent, whereas immunisation protocols using rat derived MOG35-55 peptide or recombinant rat MOG (rrMOG) are independent of B cells (Lyons *et al.*, 1999; Oliver *et al.*, 2003). MOG35-55- and rrMOG- induced EAE in B cell deficient C57BL/6 mice are indistinguishable from EAE in Wt mice with regard to disease incidence, onset, severity and CNS pathology (Lyons *et al.*, 1999). This is in line with our finding that C57BL/6 FcγR$^{-/-}$ mice are susceptible to MOG35-55 induced EAE. Delayed onset of EAE in FcγR$^{-/-}$ mice may be related to absence FcRγ chain signalling functions that are unrelated to IgG receptors. Observations on the role of B cells and FcγR in EAE in DBA/1 mice support this. B cell deficient DBA/1 mice develop EAE after immunisation with rrMOG, although clinical severity and demyelination are lower

than in Wt DBA/1 mice (Svensson *et al.*, 2002). Interestingly, the effect of FcRγ deficiency is more dramatic: EAE in FcRγ$^{-/-}$ DBA/1 mice was nearly absent (Abdul-Majid *et al.*, 2002).
The FcRγ chain associates with at least six FcR-unrelated receptor complexes in leukocytes, including the γδT cell receptor (Qian *et al.*, 1993; Koyasu, 1994; Arase *et al.*, 1997; Takai and Ono, 2001; Wu *et al.*, 2001). The role of FcRγ chain in these receptor complexes is ill defined and as consequence FcRγ$^{-/-}$ mice have been considered almost exclusively as mice lacking activating FcγR, thereby possibly ignoring less obvious deficiencies.
Earlier studies reported severely attenuated MOG35-55 EAE in B6129PF2 FcRγ$^{-/-}$ mice (Lock *et al.*, 2002; Pedotti *et al.*, 2003). The immunisation protocol was similar to ours and mice were of the same H2 haplotype (H-2^{b}), suggesting that responses to the immunisating antigen and antigen presentation may be comparable. Genetic variation in mouse strains (e.g. C57BL6/129 F2 vs. C57BL/6) probably accounts for the different results. Importantly, backcrosses of 129 and C57BL/6 mice may have unpredictable phenotypes in models of autoimmunity, presumably through epistatic interactions between 129 and C57BL/6 genes (Bygrave *et al.*, 2004). This emphasizes that comparisons between results obtained in different EAE experiments, using mouse strains of different origin, should be drawn very cautiously. Similarly, mast cell expressed FcγRIII has been proposed to play an important role in MOG35-55 EAE (Robbie-Ryan *et al.*, 2003). The data presented here, together with our unpublished results that FcγRIII$^{-/-}$ mice develop full-blown EAE, demonstrate that absence of FcγRIII on mast cells does not ameliorate MOG35-55 EAE, at least not in C57BL/6 mice.
Another aspect of murine FcγR that may be relevant to our observations is residual function of FcγRI in FcRγ$^{-/-}$ mice and possible FcRγ chain independent regulation of FcγRI (Barnes *et al.*, 2002; Beekman *et al.*, 2004). It is unknown to what extent residual FcγRI function affects the immune response in FcRγ$^{-/-}$ mice, as FcRγ$^{-/-}$ mice and FcγRI/III$^{-/-}$ mice (lacking expression FcγRI and FcγRIII, but not the FcRγ chain) showed similar responses in a model of IC-mediated nephritis (Tarzi *et al.*, 2003).
The capacity of anti-MOG antibodies to contribute to an ongoing CNS inflammatory and demyelinating response was previously demonstrated in models of antibody augmented EAE (Linington *et al.*, 1988; Morris-Downes *et al.*, 2002). The present study demonstrates that anti-MOG antibody mediated disease exacerbation is dependent on the Fc part of the injected antibody, but independent of the FcRγ chain. This implicates that the pathological effect of antibodies results from Fc-mediated complement activation. Although the role of complement activation in MOG35-55 induced EAE has been debated (Nataf *et al.*, 2000; Calida *et al.*, 2001; Reiman *et al.*, 2002), complement activation may be relevant in the context of antibody exacerbated EAE. In rats, the in vivo demyelinating potential of anti-MOG antibodies was correlated to their complement fixing capacity (Piddlesden *et al.*, 1993). Z12 mAb has high complement fixing capacity *in vitro* (Piddlesden *et al.*, 1993), possibly explaining the severe effect of Z12 mAb injection on clinical EAE in both Wt and FcRγ$^{-/-}$ mice. Deposition of C9, indicative of full complement activation, was observed in CNS lesions in association with exogenous anti-MOG antibodies, two days after injection in animals with EAE (Piddlesden *et al.*, 1993). At six days after antibody injection, C9 deposition was markedly lower, demonstrating that complement activation after antibody injection is a short term event (Linington *et al.*, 1989). This supports the idea that the rapid effect of antibody injection in both Wt and FcRγ$^{-/-}$ mice is complement mediated, although redundancy of FcγR and complement in antibody-exacerbated EAE cannot be excluded. In fact, Wt mice succumbed faster to antibody exacerbated EAE than FcRγ$^{-/-}$ mice, possibly reflecting a delayed response due to absence of FcγR mediated actions. In addition, previous studies demonstrated that complement depletion can not (Piddlesden *et al.*, 1991), or not completely

(Morris-Downes *et al.*, 2002), prevent acute exacerbation of EAE by anti-MOG mAb, whereas the present study demonstrates that deletion of the FcγR does not abolish the acute effect of anti-MOG mAb. This supports redundancy of FcγR and complement mediated pathways, as previously described in a model of antibody-dependent vitiligo (Trcka *et al.*, 2002).

Although the immediate effect of Z12 mAb on EAE severity was independent of FcγR, interactions between IgG and FcγR may contribute to a sustained effect of anti-MOG antibodies on inflammation and demyelination. On average three weeks after injection of Z12 mAb, Wt mice showed enhanced CNS demyelination and inflammation whereas FcRγ$^{-/-}$ mice did not. The half-life of IgG2a in serum is six to eight days (Roopenian *et al.*, 2003), therefore enhanced cerebellum pathology after up to at least twenty-five days after injection is unlikely to result from direct deposition of Z12 mAb. Furthermore, at the time of antibody injection, blood brain barrier damage in the cerebellum is limited in comparison with the spinal cord (Paul and Bolton, 2002). Two mice that were sacrificed two days after Z12 mAb injection for ethical reasons showed severe inflammation in the spinal cord, whereas infiltration of the cerebellum was negligible (data not shown). This suggests that Z12 mAb cannot directly reach the cerebellum parenchyma and that the effect of Z12 mAb is indirect. IgG2a complexed to a soluble protein can enhance both B and T cell responses against that protein through interactions with activating FcγR (Getahun *et al.*, 2004). Similarly, FcγR-mediated uptake of Z12 mAb opsonised myelin breakdown products, either at the site of demyelination or after capture of antigens in the periphery (de Vos *et al.*, 2002), could contribute to amplification of the immune response directed against myelin. Although it was beyond the scope of this study, it would be interesting to study MOG specific T cell responses in Wt and FcRγ$^{-/-}$ mice at different timepoints after anti-MOG antibody administration.

The data presented here may be relevant for the pathology and treatment of MS. Enhanced levels of anti-myelin antibodies in serum and CSF have been described MS patients (Warren and Catz, 1994; Egg *et al.*, 2001; Schmidt *et al.*, 2001) and in a subgroup of early MS patients, deposition of IgG and complement was observed in active demyelinating lesions. This suggests that antibody-mediated activation of complement plays a role in a subpopulation of MS patients (Lucchinetti *et al.*, 2000). In addition, intrathecal IgG production has been described as a prognostic marker for MS disease progression (Izquierdo *et al.*, 2002), suggesting that antibodies may contribute to sustained CNS inflammation and demyelination in MS.

In summary, MOG35-55 induced EAE in FcRγ$^{-/-}$ mice is delayed in onset, but otherwise indistinguishable from EAE in Wt mice. This demonstrates that activating FcγR are not essential for the initiation of CNS inflammation and demyelination although FcRγ chain mediated signal transduction may contribute to the preclinical phase of EAE. In addition, we show that anti-MOG antibodies can induce rapid exacerbation of CNS inflammation in absence of the FcγR chain, but that IgG-FcγR interactions may contribute to a sustained effect of anti-MOG antibodies on the CNS inflammatory and demyelinating response.

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Chapter 4

No association of Fcγ receptor(FcγR)IIa, FcγRIIIa and FcγRIIIb polymorphisms with MS

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Abstract

Anti-myelin IgGs occur in the cerebrospinal fluid (CSF) and serum of multiple sclerosis (MS) patients, and can induce inflammatory effector functions in leukocytes by crosslinking IgG receptors (FcγR). The efficiency of FcγR mediated inflammatory processes is affected by functional polymorphisms of three FcγR receptors (FcγRIIa, FcγRIIIa, FcγRIIIb).
The relevance of FcγR polymorphisms in MS was evaluated by studying the distribution of FcγRIIa, FcγRIIIa and FcγRIIIb genotypes in 432 MS patients and 515 healthy controls. No significant differences were found between MS patients and controls, or between subgroups of patients. We conclude that Fcγ receptor polymorphisms influence neither susceptibility nor clinical disease course of MS.

Introduction

Despite elaborate research in the last few decades, the pathogenesis of Multiple Sclerosis (MS) remains enigmatic. Genetic as well as environmental factors have been associated with the disease. It is generally assumed that T lymphocytes play an important role in the initiation of MS (Giovannoni and Hartung, 1996). In addition, humoral immune responses have been proposed to significantly contribute to the development of demyelination and axonal damage (Wingerchuk et al., 2001; Cross et al., 2001).

Abnormalities in the humoral response are seen in the majority of MS patients. Increased intrathecal production of immunoglobulins can be detected in over 90% of MS patients (Cross et al., 2001) and increased concentrations of immunoglobulins in the cerebrospinal fluid (CSF) have been associated with MS relapses (Izquierdo et al., 2002). A high B cell/monocyte ratio in the CSF was associated with rapid disease progression in a retrospective study (Cepok et al., 2001), and elevated anti-myelin antibody titres have been described in patients with active disease (Xiao et al., 1991; Warren and Catz, 1999).

Binding of auto-antibodies to myelin particles may trigger the inflammatory process by activation of complement or cross-linking IgG receptors (FcγR) on microglia and macrophages in the CNS (Linington et al., 1988; Storch et al., 1998; Kieseier et al., 1999). Interactions of immune complexes (IC) and FcγR can elicit a variety of leukocyte effector functions, including phagocytosis, antibody-dependent cytotoxicity, antigen presentation and release of inflammatory mediators (van der Pol and van de Winkel, 1998; Ravetch and Bolland, 2001). The characteristics of the inflammatory response following FcγR crosslinking are determined by the efficiency of the IgG-FcγR interaction.

The human genome encodes for three classes of leukocyte FcγR: FcγRI (CD64), FcγRII (CD32) and FcγRIII(CD16). Each FcγR class can be further divided into subclasses (FcγRIa, FcγRIb, FcγRIc; FcγRIIa, FcγRIIb, FcγRIIc; FcγRIIIa, FcγRIIIb), each subclass showing a characteristic expression pattern on leukocytes and binding capacity for IgG isotypes.

Polymorphisms in FcγRIIa, FcγRIIIa and FcγRIIIb further increase heterogeneity of the FcγR family. FcγRIIa is expressed on myeloid and lymphoid cells and exhibits a functional polymorphism in the ligand binding domain of the receptor, at amino acid position 131. Expression of either a histidine (H) or an arginine (R) greatly affects IgG binding capacity (Warmerdam et al., 1990; Maxwell et al., 1999). FcγRIIa-H131 interacts more efficiently with complexed IgG2 and IgG3 than FcγRIIa-R131 (Warmerdam et al., 1990; Parren et al., 1992). FcγRIIIa is constitutively expressed on NK cells and macrophages and exhibits a valine (V) to phenylalanin (F) substitution at amino acid 158. The FcγRIIIa-V158 allotype binds IgG1- and IgG3-containing IC more efficiently than FcγRIIIa-F158. Finally, neutrophil-restricted FcγRIIIb bears the neutrophil antigen (NA1/NA2) polymorphism. FcγRIIIb-NA1 is more efficient in binding IgG1 and IgG3 IC than FcγRIIIb-NA2 (Huizinga et al., 1990; Salmon et al., 1992; Bredius et al., 1994). Consequently, FcγRIIa-H131, FcγRIIIa-V158 and FcγRIIIb-NA1 induce leukocyte effector functions more efficiently than their counterparts.

Interindividual differences in the efficiency of FcγR induced inflammation may be associated with differences in susceptibility to antibody-mediated disease. Indeed, case-control studies

have documented skewed distributions of FcγRIIa, FcγRIIIa, and FcγRIIIb polymorphisms in patients with autoimmune and infectious diseases (van der Pol and van de Winkel, 1998). Myhr et al. (1999) reported an association between FcγR genotypes and MS disease course in 136 Norwegian MS patients (Myhr et al., 1999).

In this study, we evaluate the relevance of genetic FcγR heterogeneity for MS susceptibility and disease course in a large group of Dutch MS patients. We determined the distribution of FcγRIIa, FcγRIIIa, and FcγRIIIb genotypes in 432 MS patients and 515 ethnically matched healthy controls.

Materials and Methods

Subjects

A total of 432 Dutch Caucasian patients with clinically definite MS were recruited from the outpatient clinic of the Department of Neurology at VU Medical Centre (VUmc) in Amsterdam (Schrijver *et al.*, 1999). The study was carried out with the approval of the Medical Ethical Committee of the VUmc and informed consent was obtained from all subjects. All patients were seen at regular intervals at the outpatient clinic. During these visits, patient characteristics were obtained in a standardized way. Disability was expressed using the expanded disability status scale (EDSS) (Kurtzke, 1983). In addition, patient files were scrutinized to collect data on the time to reach permanent need for walking assistance (EDSS6). The latter was used to evaluate disease progression. Disease duration was determined as the interval between onset of disease and latest visit at the clinic. All patient characteristics were obtained by investigators who where at the time blind to the aim of the present study.
Five hundred fifteen ethnically matched healthy blood donors were recruited as controls.

Determination of FcγR genotypes

Genomic DNA was extracted from whole blood using standard phenol-chloroform isolation procedures. FcγR genotypes were determined by means of PCR amplification methods. FcγRIIa genotyping was performed using two PCR techniques. All controls were genotyped as described by Carlsson et al. (1998) (Carlsson *et al.*, 1998). In short, a 1000bp product encompassing the polymorphic site was amplified using FcγRIIa specific primers. This reaction product was then used as a template in two separate reactions, using allele-specific primers. In addition, all patients and 50 control subjects were FcγRIIa genotyped using a modified allele-specific reaction as described by Smyth et al. (1997) (Smyth *et al.*, 1997). In short, approximately 100ng of DNA was added to a 50 µl reaction mix containing 50mM KCl, 15mM Tris-HCl, 1mM dNTPs (Invitrogen), 3mM $MgCl_2$, 2 U Ampli taq Gold (Perkin Elmer, Foster City, CA), and 10 pmol of the following primers: 5'-*ctg aaa aac cct tgg aat c*-3', 5'-*tct cag acc tcc atg tag*-3', and either 5'-*aat ccc aga aat tct ccc g*-3' (FcγRIIa-R131 specific reaction) or 5'-*aat ccc aga aat tct ccc a*-3' (FcγRIIa-H131-specific reaction). Amplification was initiated by incubation at 95°C for 10 minutes, followed by 35 cycles of 95°C for 20 seconds, 56°C for 30 seconds and 72°C for 15 seconds. PCR reactions were terminated by incubation at 72°C for 7 minutes. To test accuracy of these assays, 50 control subjects were genotyped using both techniques, yielding identical results. FcγRIIIa genotyping was performed in two separate reactions using allele-specific primers as previously described (Leppers-van de Straat *et al.*, 2000). FcγRIIIb genotyping was performed in two separate reactions using allele-specific primers as previously described (De Haas *et al.*, 1995) with minor modifications. In short, 100ng of DNA was added to a 50 µl reaction mix containing 50mM KCl, 15mM Tris-HCl, 1mM dNTPs (Invitrogen), 3mM $MgCl_2$, 2 U AmpliTaq Gold, and either 20pmol NA1 specific or 10pmol of NA2 specific primers. FcγRIIa flanking primers (7.5pmol) amplifying a 406 bp fragment were used as internal controls, to ensure validity of negative PCR results. Samples were subjected to a hot start and one-cycling procedure: 10 min at 95°C followed by 35 cycles (30 sec at 95°C, 30 sec at 57°C and 30 sec at 72°C) and terminated by incubation for 7 min at 72°C.

In all experiments, DNA from both homozygous and the heterogeneous genotypes (as verified by automatic sequencing) were included as internal controls. PCR products were loaded on 2% (FcγRIIa and FcγRIIIb) or 3% (FcγRIIIa) agarose gels containing ethidium bromide and visualized with a Photo Imager (Uppsula, Sweden).

Statistics

Chi-square test and 3 x 2 contingency tables were used for comparison of FcγR genotype distributions between groups. Kaplan Meijer analysis and log-rank tests were used to analyse differences in disease progression between FcγR genotypes. Average time to EDSS6 was compared between patients with different FcγR genotypes using Mann-Whitney and Kruskal-Wallis tests. Mann-Whitney test was used to statistically analyse the age of onset of MS between genotypes. Significance was set at p-values < 0.05.

Results

Patient characteristics

Patient characteristics are summarised in Table 1. Females accounted for 270 of the patients. All patients were unrelated, Caucasian, and from European-Dutch descent. One hundred seventy-eight patients had relapsing-remitting (RR) MS, 159 had secondary progressive (SP) MS and 95 had primary progressive (PP) MS. Average disease duration of the patient population was 13.4 years, median disease duration was 12 years. Disease duration ranged from 0.2 to 63.0 years.

Table 1. Clinical characteristics of MS patients included in the study

		No. of patients (female/male)	Disease duration (years)		
			Mean	Median	Range
MS subtype[a]	RR	178 (121/57)	10.0	9	0.2 - 33.3
	SP	159 (94/65)	17.1	15	4.0 - 63.0
	PP	95 (55/40)	13.6	13	2.3 - 44.2
Total		432 (270/162)	13.4	12	0.2 - 63.0

[a]MS clinical subtypes: RR = relapsing remitting, SP = secondary progressive, PP = primary

Genotype distributions in MS patients and healthy control subjects

Genes encoding FcγRIIa, FcγRIIIa and FcγRIIIb were screened for polymorphisms using allele-specific primers in a PCR reaction. Genotypes of control subjects were in Hardy-Weinberg equilibrium for all three FcγR (data not shown).

FcγRIIa genotypes were obtained of 431 patients. Table 2A shows the distribution of FcγRIIa genotypes and allele frequencies in MS patients, clinical subgroups of MS patients and healthy donors. FcγRIIa genotypes frequencies of MS patients closely resembled the genotype frequencies of healthy donors. Similar results were obtained when subgroups of MS patients (RR, SP and PP) were compared separately to healthy controls. In addition, no significant differences were observed when the three MS subgroups were compared to each other.

FcγRIIIa genotypes were obtained of 422 patients (table 2B). Distribution of genotypes in MS patients was similar to the distribution in healthy donors. Furthermore, FcγRIIIa genotype distributions did not differ between MS subtypes.

Table 2C shows FcγRIIIb genotype distribution. Genotypes could be determined in 418 MS patients. Again, no differences between patients, subgroups of patients and healthy controls were observed.

We next evaluated combinations of FcγRIIa-IIIa, FcγRIIa-IIIb, and FcγRIIIa-IIIb genotypes in patients and controls. No significant differences were found between MS patients and healthy donors (data not shown).

FcγR genotype and age at onset of disease

The age of disease onset was compared between MS patients, stratified for FcγR genotypes. No differences were observed between FcγR genotypes (data not shown).

Table 2. Distribution of FcγRIIa (A), FcγRIIIa (B) and FcγRIIIb (C) genotypes in MS patients and healthy donors

A		FcγRIIa genotype – n (%)			Allele frequency	
		RR	RH	HH	R	H
MS patients		107 (24.8)	199 (46.2)	125 (29.0)	0.48	0.52
MS subtypes	RR	40 (22.6)	84 (47.5)	53 (29.9)	0.46	0.54
	SP	44 (27.7)	74 (46.5)	41 (25.8)	0.51	0.49
	PP	23 (24.2)	41 (43.2)	31 (32.6)	0.46	0.54
Healthy donors		124 (24.1)	256 (49.7)	135 (26.2)	0.49	0.51

B		FcγRIIIa genotype – n (%)			Allele frequency	
		VV	VF	FF	V	F
MS patients		68 (16.1)	197 (46.7)	157 (37.2)	0.39	0.61
MS subtypes	RR	24 (13.6)	90 (50.8)	63 (35.6)	0.39	0.61
	SP	28 (18.3)	61 (39.9)	64 (41.8)	0.38	0.62
	PP	16 (17.4)	46 (50.0)	30 (32.6)	0.42	0.58
Healthy donors		74 (14.4)	244 (47.4)	197 (38.2)	0.38	0.62

C		FcγRIIIb genotype – n (%)			Allele frequency	
		NA1NA1	NA1NA2	NA2NA2	NA1	NA2
MS patients		51 (12.2)	192 (45.9)	175 (41.9)	0.35	0.65
MS subtypes	RR	21 (11.9)	76 (43.2)	79 (44.9)	0.34	0.66
	SP	18 (11.9)	73 (48.4)	60 (39.7)	0.36	0.64
	PP	12 (13.2)	43 (47.2)	36 (39.6)	0.37	0.63
Healthy donors		68 (13.2)	240 (46.6)	207 (40.2)	0.37	0.63

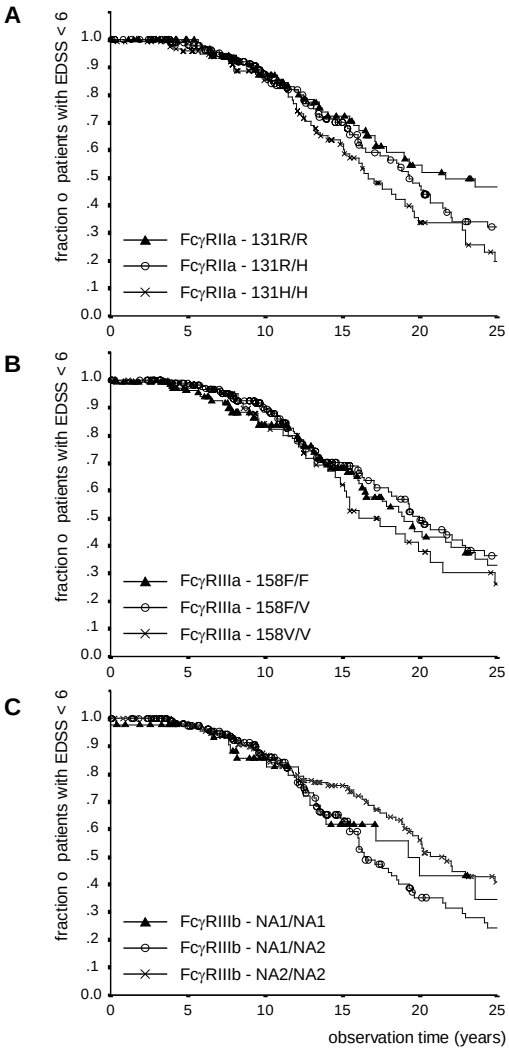

Figure 1.

Kaplan-Meier analysis of time to reach Expanded Disability Status Scale score 6.0 after disease onset. MS patients were stratified according to their FcγRIIa (A), FcγRIIIa (B) or FcγRIIIb (C) genotype.

FcγR genotype and disease progression

The interval between disease onset and moderate to severe disability (EDSS 6) was used as an estimate for disease progression. At the time these data were obtained, 163 patients had reached an EDSS of 6 or higher. Two-hundred and two patients (47% of total) were followed for 12 years or longer. In the total patient population, the median time to EDSS6 was 9.0 years, ranging from 0.4 to 56 years (average 10.4, standard deviation 7.9). We compared average time to EDSS6 for patients expressing different FcγR genotypes using ANOVA, Kruskal-Wallis and Mann-Whitney tests. No differences were found for any of the three FcγR polymorphisms (data not shown). In addition, Kaplan-Meijer analysis did not show significant differences in disease progression between FcγR genotypes (Figure 1A-1C).

Discussion

We compared the distribution of polymorphisms in the genes encoding for FcγRIIa, FcγRIIIa and FcγRIIIb in a large group of Dutch MS patients and ethnically matched controls. FcγR genotype distribution in the healthy donor population was similar to what was previously described for Caucasians from the Netherlands, Germany and the United States (van der Pol and van de Winkel, 1998; van Schie and Wilson, 2000). FcγR polymorphisms could not be associated with disease susceptibility, and the distribution of FcγR genotypes was similar in all subgroups of MS patients. In addition, FcγR genotypes did not correlate with disease progression or age of disease onset. We cannot exclude correlations with other disease parameters that were not tested (e.g., exacerbation frequency). However, these data show that it is unlikely that FcγRIIa, FcγRIIIa and FcγRIIIb genotypes play a significant role in disease susceptibility or modification of the long term clinical course of MS in Dutch patients.
Clinical relevance of interindividual FcγR heterogeneity has been suggested for both infectious and autoimmune diseases. Relatively inefficient handling of IC by FcγRIIa-R131, FcγRIIIa-F158 and FcγRIIIb-NA2 is proposed to favour the persistence of bacterial infections or the deposition of pathogenic IC, thus causing prolonged pro-inflammatory reactions. This may explain the reported association of FcγR genotypes with diverse ailments such as meningococcal and pneumococcal infections (Sanders *et al.*, 1994; Bredius *et al.*, 1994; Yee *et al.*, 2000; van der Pol *et al.*, 2001), SLE (Duits *et al.*, 1995; Salmon *et al.*, 1996) and RA (Morgan *et al.*, 2000).
Thus far, one study focussed on FcγR polymorhpisms in MS. One hundred thirty-six Norwegian patients were compared to 96 healthy donors (Myhr *et al.*, 1999). No differences were found in the distribution of FcγRIIa and FcγRIIIb polymorphisms between patients and controls. However, FcγRIIIb genotypes were found to be associated with disease course. Patients homozygous for FcγRIIIb-NA1 were reported to have significantly better outcome of disease as measured by EDSS, than those who were heterozygous or homozygous for NA2. In addition, disease course in FcγRIIa-H131 homozygotes was reported to be more favourable than in heterozygotes or patients homozygous for FcγRIIa-R131. We could not reproduce these findings in the present study.
Comparisons between geographically separate populations may be complicated by ethnical differences in the genetics of control subjects. FcγRIIa-H131 and –R131 allele frequencies were 0.42 and 0.58 respectively among healthy Norwegian donors, compared to 0.51 and 0.49 in the present study. As a consequence, the distribution of FcγRIIa genotypes over healthy controls was different in the two populations. In contrast, FcγRIIIb-NA1/NA2 allele frequencies and genotype distributions were very similar among Dutch and Norwegian controls (0.36/0.64 and 0.37/0.63 respectively). Thus, in this case genetic differences between control populations should not interfere with comparison of the two.
Several issues may account for the different results. First, the total number of subjects in the study by Myhr *et al*. (1999) is smaller than in the present study. The number of subjects required to detect small differences in the distribution of genetic polymorphisms is very large (Ebers and Dyment, 1998), and conclusions from smaller studies should be drawn with great

care. It is possible that the association described by Myhr *et al*. (1999) is occasional, and the result may not be confirmed when larger groups of Norwegian MS patients and healthy donors are compared.

Another issue is the correction for multiple testing. Because of the increased likelihood of type II errors it can be argued to renounce correction for multiple testing in exploratory studies. This of course increases the risk of type I errors, which might have occurred in the study by Myhr *et al*. (1999). Despite the fact that we performed multiple tests and analysed the polymorphisms separately, we did not observe any significant effect at the 0.05 cut-off level. This seems to indicate the robustness of our negative findings in this large sample of patients and controls.

Conflicting reports on the role of immunoglobulins and FcγR in MS patients may be the result of the heterogeneous nature of MS. Disease course can be very different among patients, and MS lesion formation may differ from one patient to another. Importantly, Lucchinetti and Lassman proposed a new way to classify MS lesions (Lucchinetti *et al.*, 2000), based on immunohistochemical analysis. They suggest that demyelination and axonal damage follow different pathogenetic pathways in subgroups of patients. MS lesions were classified by the presence of different cell types, immunoglobulin deposits and damage to neurons and glia cells. Type II lesions, as described by Lucchinetti *et al*. (2000) are characterized by deposition of immunoglobulins and activated complement at sites of active myelin destruction. It is in these lesions that polymorphisms in FcγR could affect lesion formation. Unfortunately, the new classification of MS lesions (Lucchinetti *et al.*, 2000) does not correspond with the classical subdivision of MS based on disease course (primary progressive, relapsing-remitting and secondary progressive). The subdivision of MS patients used here may therefore not be the most desirable for this study.

The most obvious explanation for our results is probably that FcγR polymorphisms simply do not affect MS susceptibility or MS disease course, or at least not to a measurable extent. Both immune complex-complement mediated processes and FcγR mediated processes may contribute to antibody-related inflammation in the MS brain. FcγR polymorphisms may affect the balance of these two processes in individual MS patients. However, both processes will eventually lead to augmentation of the inflammatory response. Thus, even though local inflammatory processes may differ among patients as a result of FcγR polymorphisms, these differences may not be reflected at the level of systemic disease progression.

In summary, functional polymorphisms in the genes encoding for FcγRIIa, FcγRIIIa and FcγRIIIb could not be associated with MS susceptibility or disease course. Allele frequencies for all genes were highly similar in MS patients and healthy donors. We conclude that FcγR polymorphisms do not form a genetic risk marker for MS.

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Chapter 5

Complement and IgG are consistently associated with active demyelination in MS

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Submitted

Abstract

Complement activation and IgG deposition have been described in active demyelinating lesions in patients with an acute or tumour-like presentation of MS. Absence of complement and IgG from other MS patients suggested heterogeneity in the pathogenesis of demyelination. The aim of this study was to characterize complement activation, IgG deposition and Fcγ receptor (FcγR) expression in active demyelinating lesions in an unselected autopsy material of MS patients. Post-mortem tissue of 32 MS patients containing 97 lesions was studied using immunohistochemistry. Complement and IgG were consistently observed on and within macrophages in active demyelinating areas. Immunostaining for complement and IgG was infrequent or absent in later lesion stages and in control white matter. Macrophages in active demyelinating areas were also immunopositive for FcγR. Double-labelling studies revealed colocalisation of complement, IgG and FcγR with myelin proteins in macrophages, suggesting an important role for complement- and FcγR-mediated myelin phagocytosis in established MS. In active demyelinating areas enhanced production of complement was detected using quantitative PCR. Heterogeneity between MS patients was not observed with regard to complement and IgG in active demyelinating areas. This indicates that the immunopathological mechanisms of white matter demyelination are different in established MS than in the MS subpopulations studied earlier.

Introduction

Multiple sclerosis (MS) is a chronic demyelinating disease of the central nervous system (CNS), resulting in progressive loss of motor and sensory function. Characteristic for MS are focal areas (lesions or plaques) of myelin and partial axonal loss within the CNS parenchyma. Predilection sites are optic nerve, periventricular white matter, subpial cerebral cortex, brainstem and cervical spinal cord (Lumsden CE, 1970; Bö *et al.*, 2003).

It is not known what initiates demyelination in MS lesions. Autoimmune inflammation mediated by infiltrating T and B cells specific for myelin antigens may result in recruitment of macrophages and removal of healthy myelin (Adams *et al.*, 1989), alternatively death or damage of oligodendrocytes may be a primary event followed by (auto-immune) inflammation and demyelination (Barnett and Prineas, 2004). One study suggests that both these mechanisms exist, although in different subsets of acute MS patients (Lucchinetti *et al.*, 2000). In one subset of MS patients, active demyelinating lesions were characterized by the presence of activated complement and antibodies, while other patients showed inflammatory lesions without complement and immunoglobulins, or signs of primary oligodendrocyte pathology (Lucchinetti *et al.*, 2000).

All of the suggested pathogenic pathways eventually lead to the removal of myelin by macrophages. EM studies demonstrated that in active demyelinating MS lesions macrophages lift myelin off axons and contain myelin fragments intracellularly in coated pits, suggesting a receptor-mediated phagocytic process (Prineas and Connell, 1978). This may occur through several mechanisms, including Fcγ receptor (FcγR) and complement receptor mediated phagocytosis (Smith, 1999). *In vitro* experiments indicate that complement components and antibodies can contribute to myelin phagocytosis both independently and synergistically. Direct binding of C1q and C3 to myelin respectively activates the classical and alternative pathways of complement. Subsequent formation of the membrane attack complex (MAC) results in fragmentation of myelin, facilitating myelin uptake (DeJong and Smith, 1997). In addition, C3-opsonized myelin is efficiently taken up by macrophages and microglia through complement receptor 3 (CR3) (Bruck and Friede, 1991; van der Laan *et al.*, 1996). Immunoglobulins specific for myelin antigens enhance myelin phagocytosis through interactions with FcγR (Abdul-Majid *et al.*, 2002). FcγR mediated phagocytosis is further enhanced in the presence of fresh serum, indicating an additional role for complement and complement receptors (Van der Goes *et al.*, 1999), presumably through antibody-dependent activation of the classical pathway. It is unclear to what extent each of these mechanisms contributes to demyelination in MS plaques.

Several studies characterized the presence and expression of complement, IgG and Fcγ receptors in MS. All three subclasses of Fcγ receptors, FcγRI (CD64), FcγRII (CD32) and FcγRIII (CD16), are constitutively expressed by ramified microglia and perivascular macrophages in the CNS (Ulvestad *et al.*, 1994). FcγR expression is enhanced on lipid-laden macrophages in active MS lesions (Ulvestad *et al.*, 1994), but it is unknown if these FcγR are involved in myelin phagocytosis. Interestingly, Prineas and Graham (Prineas and Graham, 1981) showed capping of phagocytic macrophages with IgG in MS lesions, suggestive of

antibody mediated phagocytosis. However, colocalisation of FcγR with IgG has not been described in MS lesions.

Deposition of complement and IgG in white matter MS lesions was reported by a number of groups. In addition to the diffuse immunostaining in active MS lesions that probably results from leakage of complement proteins through a damaged blood brain barrier (BBB), complement activation products and IgG were found in capillary walls in active MS plaques (Compston *et al.*, 1989; Gay and Esiri, 1991). Direct deposition of complement and IgG on myelin sheaths or degraded myelin has been described as well, although less consistently (Storch *et al.*, 1998; Lucchinetti *et al.*, 2000; Bruck *et al.*, 2001; Barnett and Prineas, 2004). In subgroups of MS patients, complement and IgG have been detected on and within phagocytic macrophages in areas of active demyelination (Gay *et al.*, 1997; Lucchinetti *et al.*, 2000).

Importantly, the results mentioned above were obtained almost exclusively using biopsy or autopsy material taken from patients with acute MS or acute disease exacerbations, usually with short disease duration. It is unclear to what extent deposition of complement and IgG are found in active demyelinating lesions of an unselected autopsy material of patients with established MS, and whether such deposition is heterogeneous in this patient group.

Another unresolved issue is the origin of complement in MS lesions. Deposition of complement is observed predominantly in active lesions, where the BBB is disrupted and serum proteins can easily diffuse into the CNS parenchyma (Gay and Esiri, 1991). However, all resident brain cells are capable of producing complement components (Barnum, 1995). Local production of complement is increased in a number of neurological diseases (Veerhuis *et al.*, 1996; Singhrao *et al.*, 1999; Grewal *et al.*, 1999), but it is unknown if local production plays a role in MS.

The aim of this study was to characterize presence and distribution of complement and IgG in active demyelinating lesions in an unselected MS patient material with a typical course of disease, and to investigate whether heterogeneity exists between MS patients with regard to the deposition of complement and IgG. In the same material, the expression of FcγR was analysed. In addition, local production of complement was assessed in autopsy tissue containing areas of active demyelination.

Materials and Methods

Autopsy procedures and classification of lesion areas

Post mortem material from MS patients was obtained through cooperation with the Dutch Brain Bank (coordinator: Rivka Ravid). The MS diagnosis was clinically and neuropathologically confirmed in all patients. MS lesions containing areas of active demyelination are relatively rare; therefore all MS tissue specimens present at the Department of Pathology of the VU Medical Centre were checked for the presence of such lesions.

The material encompassed paraffin embedded tissue specimens from 66 MS patients, containing 348 white matter lesions. MS lesions were obtained using MRI guided sampling as described previously (Bö *et al.*, 1994; de Groot *et al.*, 2001). Briefly, at autopsy 1 cm thick tissue slices were used for MRI. After MRI, the tissue slices were cut in two parallel to the surface. From one half tissue blocks containing MS lesions were fixed in formalin and embedded in paraffin, whereas corresponding blocks from the opposing half were snap frozen and stored in liquid nitrogen. The paraffin blocks were used to identify tissue blocks containing active demyelinating MS lesions (see below). Corresponding frozen tissue blocks were subsequently used in the immunohistochemical study of complement, IgG and FcγR in MS.

Post mortem paraffin embedded tissue blocks were stained as described before with antibodies directed against HLA-DR and PLP to characterize lesion inflammatory and demyelinating activity (Bö *et al.*, 1994). The inflammatory activity of lesions was staged as active, chronic active, or chronic inactive as described (Bö *et al.*, 1994; Trapp *et al.*, 1998). Demyelinating activity was defined by the presence of macrophages containing intracellular PLP (Lassmann *et al.*, 1998). As lesion activity can be variable in different areas of one lesion (for instance in chronic active lesions), centre and border areas of MS lesions were analysed separately.

To combine classification of inflammatory and demyelinating activity within MS lesions, areas containing HLA-DR$^+$ macrophages with intracellular staining for PLP were classified as inflammatory demyelinating (HLA-DR$^+$ PLP$^+$), areas containing HLA-DR$^+$ macrophages in absence of intracellular PLP were classified as inflammatory non-demyelinating (HLA-DR$^+$ PLP$^-$) and areas containing few macrophages with low HLA-DR staining and no intracellular PLP were classified as inactive (HLA-DR$^-$ PLP$^-$). Importantly, this method of classification allows identification of different activity levels within one lesion. For example, a chronic active MS lesion may contain an inflammatory demyelinating rim and an inactive centre.

Frozen tissue blocks, corresponding to paraffin blocks containing at least one inflammatory demyelinating area, were used for further study, as previously MS lesion heterogeneity was described in active demyelinating areas only (Lucchinetti *et al.*, 2000). This constituted 28 tissue specimens from the CNS white matter of 16 MS patients. In order to study sufficient numbers of lesions with different inflammatory and demyelinating activity, 6 additional tissue specimens from the same patients and 18 tissue specimens from 16 other MS patients were included.

Table 1. Clinical characteristics of MS patients and control subjects included in the study

Case	Age (y)	MS subtype	Disease duration (y)	Sex	Cause of death	Postmortem delay (h)
MS1	57	PPMS	22	f	Sepsis	05:45
MS2	35	SPMS	11	f	Cachexia/general decline	05:35
MS3	40	SPMS	14	f	Dehydration	07:00
MS4	53	SPMS	18	f	Pneumonia	07:16
MS5	62	SPMS	29	f	Cardiac asthma	06:45
MS6	46	SPMS	23	m	Pneumonia	03:35
MS7	40	SPMS	11	f	Aspiration pneumonia	07:00
MS8	54	MS	37	f	Pneumonia/UTI	07:00
MS9	55	SPMS	11	f	Aspiration pneumonia	07:35
MS10	70	PPMS	19	f	Cardiogenic shock; pneumonia	08:55
MS11	45	SPMS	14	f	Euthanasia	10:55
MS12	58	SPMS	20	f	Euthanasia	08:10
MS13	71	RRMS	24	f	Respiratory insufficiency	10:25
MS14	38	RRMS	14	f	Sudden death, cause unknown	05:15
MS15	70	MS	21	m	Unknown	06:25
MS16	52	SPMS	22	f	Pneumonia	08:25
MS19	64	PPMS	34	m	End stage MS	07:30
MS20	48	SPMS	8	f	Euthanasia	08:10
MS21	66	SPMS	43	f	Cancer; liver failure	6:20
MS22	53	SPMS	23	f	Euthanasia	10:45
MS23	43	SPMS	17	m	Pneumonia	8:30
MS24	81	PPMS	51	m	General deterioration	08:50
MS25	75	SPMS	42	F	Pneumonia	08:00
MS26	77	PPMS	26	m	Stroke	04:15
MS27	71	SPMS	23	f	Respiratory problems	10:15
MS28	48	SPMS	25	f	Euthanasia	04:50
MS29	72	SPMS	13	f	Pneumonia	12:00
MS30	55	PPMS	19	f	Possible stroke	17:00
MS31	49	MS	21	f	Breast cancer	05:45
MS32	84	MS	49	f	Euthanasia	08:45
CTRL1	59			f	Larynx carcinoma	06:20
CTRL2	45			f	Adenocarcinoma	13:30
CTRL3	52			f	Leiomyosarcoma	06:50
CTRL4	70			m	Unknown	04:40
CTRL5	50			f	Suicide	14:35
CTRL6	60			m	Myocardial infarction	8:00

In total 58 tissue blocks from 32 MS patients were thus available for further study. In addition, 5 white matter tissue specimens from 5 non-neurological control subjects were studied.

Patient characteristics

The average age of the 32 MS patients included in the study was 57.3 years (± 13.9 years). The median disease duration was 22.0 years (range 8-51 years). Two patients had relapsing remitting MS (RRMS), 19 patients had secondary progressive MS (SPMS), 6 patients had primary progressive MS (PPMS) and of 5 MS patients the clinical subtype was unknown. Of all patients, 25 were female. The average age of the non-neurological control subjects at time of death was 56.0 years (± 9.7 years). Four control subjects were female. Detailed patient characteristics are provided in table 1.

Immunohistochemical methods

Five µm cryostat sections were mounted on Poly-l-lysine coated glass slides, fixed in acetone (10 min), washed in phosphate buffered saline (PBS; pH 7.4) and pre-incubated with normal serum (swine serum 1:10, rabbit serum 1:50 diluted in 1% bovine serum albumin in PBS for 10 minutes. Frozen sections were incubated for 60 minutes at room temperature (RT) or overnight (4°C) with primary antibodies directed against HLA-DR, PLP, complement components, IgG, FcγR and fibrinogen (table 2).

The tissue sections were then incubated with a biotinylated swine-anti-rabbit $F(ab')_2$ (1:300, DakoCytomation, Glostrup, Denmark) or a biotinylated rabbit-anti-mouse $F(ab')_2$ (1:500, Dako) for 30 minutes at RT. Sections were incubated for 1 hour with the s-ABC-HRP complex (1:200, Dako), peroxidase labelling was visualized by 3,3-diaminobenzidine (Sigma, St. Louis MO). For sections immunostained with the EnVision method, the sections were incubated with the EnVision-HRP complex (undiluted, Dako) after primary antibody. The tissue sections were counterstained with haematoxylin. As negative controls, PBS (pH 7.4), irrelevant polyclonal antibody and isotype specific control monoclonal antibodies (IgG1, IgG2a, IgG2b) were used instead of primary antibodies. Negative controls were essentially blank. Alzheimer's disease autopsy brain tissue was used for positive controls for the complement immunohistochemistry.

For double labelling studies using immunofluorescence, the sections were incubated with a mixture of biotinylated swine-anti-rabbit $F(ab')_2$ (1:300, DAKO) and HRP-conjugated goat-anti-mouse isotype specific secondary antibodies, after primary antibody (table 2). The sections were then incubated with streptavidin conjugated to $Alexa^{488}$ (1:750, Molecular Probes) for 60 min and rhodamin-tyramide (1:3000 + 0.01% H_2O_2, VUmc) for 5 min. If the primary antibodies used in the double labelling studies were of the same species and isotype, staining for the antigens of interest, including detection using $Alexa^{488}$- and $Alexa^{594}$-conjugated secondary antibodies, was performed sequentially on the same section. As negative controls, PBS (pH 7.4), irrelevant polyclonal antibody and isotype control monoclonal antibodies were used instead of primary antibodies. Negative controls were essentially blank. In addition, single

fluorescent labelling of the primary antibodies was used on MS brain tissue to verify the staining pattern compared to the double labelling techniques.

Scoring of immunopositivity

The presence and extent of immunopositivity for complement activation products (C1q, C3d, C4d and C5b-9), fibrinogen, IgG, FcγRI, FcγRII and FcγRIII on macrophages, astrocytes, myelin and blood vessels was scored semi-quantitatively in the different lesion areas (centre and border of white matter lesions, normal appearing white matter (NAWM) of MS patients and controls), using a semi-quantitative scale from (-) to (+++). The density of stained cells/structures and the intensity of staining of each individual cell/structure were taken into consideration. (-) represented no immunostaining, (+) light immunopositivity, (++) moderate immunostaining and (+++) strong immunopositivity. Immunopositivity was analysed separately on macrophages, myelin, astrocytes and blood vessels in centre and border areas of white matter lesions.

Table 2 Primary antibodies used for immunohistochemistry

Antigen	Species/isotype	Clone	Dilution	Company	Method
C1q	Rabbit polyclonal		1:1000	DAKO	s-ABC-HRP
C3d	Rabbit polyclonal		1:500	DAKO	s-ABC-HRP
C4d	Mouse IgG1		1:100 (o/n)	Quidel	s-ABC-HRP
C5b-9	Mouse IgG2a	aE11	1:50 (o/n)	DAKO	EnVision
C5b-9	Mouse monoclonal	B7	1:100 (o/n)	Gift Dr.Morgan	EnVision
FcγRI(CD64)	Mouse IgG1	10.1	1:20	Serotec	s-ABC-HRP
FcγRII(CD32)	Mouse IgG1	AT10	1:750	Serotec	s-ABC-HRP
FcγRIII(CD16)	Mouse IgG1	3G8	1:500	Gift Dr. Leusen	s-ABC-HRP
Amyloid-β	Rabbit polyclonal		1:600	Gift Dr. Nostrand	s-ABC-HRP
Fibrinogen	Rabbit polyclonal		1:100	DAKO	s-ABC-HRP
GFAP	Mouse IgG1	6F2	1:10	Monosan	s-ABC-HRP
HLA-DR	Mouse IgG2b	LN3	1:100 (o/n)	Gift Dr.Hilgers	s-ABC-HRP
IgG	Rabbit polyclonal		1:800	DAKO	s-ABC-HRP
PLP	Mouse IgG2a	Plpc1	1:1000 (o/n)	Serotec	s-ABC-HRP

GFAP = glial fibrillary acidic protein; PLP = proteolipid protein; o/n = overnight incubation; DAKO = DakoCytomation, Glostrup, Denmark; Quidel = Quidel Corporation, San Diego, CA; Serotec = Serotec Oxford, UK; Monosan = Monosan, Uden, The Netherlands

Quantitative PCR

Tissue specimen containing inflammatory demyelinating lesion areas from 5 MS patients were selected for quantitative PCR (Q-PCR) to estimate the relative expression of complement mRNA. Tissue specimens from 4 non-neurological controls were used as controls (CTRL2-4, CTRL6). From each tissue block, four 10 μm sections were lysed in TRIzol (Life Technologies, Gaithersburg, MD), according to the manufacturer's guidelines. Chloroform (0.2ml/ml lysate) was added and lysates were mixed vigorously for 30 s. The mixture was centrifuged (12000 rpm, 4ºC, 15 min), and the aqueous phase was taken up in a new tube. RNA was precipitated using 0.5ml isopropanol and incubated for 1 h at -20ºC. After centrifugation (12000 rpm, 4ºC, 10 min) the supernatant was removed and pellets were washed using 75% EtOH, again followed by centrifugation (4000 rpm, 4ºC, 5 min). The supernatant was removed and pellets were allowed to dry for 10 min. RNA was resuspended in 40μl diethylpyrocarbonate (DEPC) treated H_20 and stored at -80ºC. After treatment with DNAse (AppliChem, Darmstadt, Germany) to digest genomic DNA, RNA was reverse transcribed using a CDNA synthesis kit (MBI Fermentas, St. Leon-Rot, Germany). Real-time quantitative PCR was performed using an Abiprism 7900 Sequence Detection System (PE Applied biosystems, CA, USA) based on specific primers and general fluorescence detection with SYBR green. Cyclophilin A and GAPDH were used to control for sample loading and to allow normalization between samples. Primer sequence: GAPDH, sense: 5' ACCTGACCTGCCGTCTAGAAAA 3', anti-sense 5' GCCCAGGATGCCCTTGA 3'; cyclophilin A sense: 5' TTTCATCTGCACTGCCAAGACT 3', antisense: 5' CCATTCCTGGACCCAAAGC 3'; C1q sense: 5' CCCCGATGGCCAACCT 3', anti-sense 5' GCCGACTTTTCCTGGATTCC 3', C3 sense 5' ACAGCAGCGCACGTTCATC 3', anti-sense 5' ACCCAAGTGTCCTTCCCGA 3'.

Statistical methods

Differences in immunopositivity between lesion areas were calculated using non-parametric tests (Mann Whitney U-test, Sign Test). Correlations of immunoreactivity between groups were calculated using the Spearman rank test. Local production of complement mRNA in MS tissue specimen was considered to be enhanced over production in non-neurological control tissue when the relative abundance of mRNA in MS tissue was higher than [average abundance of non-neurological control tissue + 2*standard deviation]. All statistical analyses were performed 2-tailed with a confidence level of 95% or higher when indicated.

Results

Number of lesions and areas

The frozen tissue specimens from 32 MS patients contained a total of 97 MS lesions, as revealed by immunostaining for PLP and HLA-DR. Centre and border areas of the lesions were studied separately, yielding a total of 194 areas. The material comprised 81 inflammatory demyelinating areas, 62 inflammatory non-demyelinating areas and 51 inactive areas. Of all inflammatory demyelinating areas, 44 areas were part of active MS lesions and 37 were part of chronic active lesions.

Macrophages in areas of active demyelination contain activated complement and IgG

Generally, macrophage immunopositivity for complement activation products, IgG and fibrinogen was associated with inflammatory demyelinating areas. In addition to staining on the cell surface, complement, IgG and fibrinogen showed extensive intracellular immunostaining in vesicular structures, a pattern highly similar to the cellular pattern of PLP immunopositivity within the same areas (figure 1A,C,G,E). Deposition of complement, IgG and fibrinogen in inflammatory non-demyelinating areas or inactive areas was generally much lower or absent (figure 1B,D,F,H). Results of semi-quantitative analysis of macrophage-associated immunostaining are provided in figure 2.

C1q and C3d were consistently found on and within macrophages in inflammatory demyelinating areas, but not in inflammatory non-demyelinating areas, inactive areas and NAWM (for both C1q and C3d, inflammatory demyelinating areas vs. any other area $p<0.001$). Immunostaining for C4d on and within macrophages was less abundant and less intense than for the other complement activation products, but again the highest extent of immunopositivity was observed in inflammatory demyelinating areas. Immunostaining for the terminal complex of complement activation (C5b-9) yielded similar results with the two different monoclonal antibodies used. C5b-9 immunopositivity was abundantly present on and within macrophages in inflammatory demyelinating areas (figure 1E). Although there was considerable immunopositivity for C5b-9 in inflammatory non-demyelinating areas, staining was significantly less than in inflammatory demyelinating areas ($p<0.05$). C5b-9 immunostaining in inactive areas and NAWM was low (figure 2).

Macrophage associated immunostaining for IgG and fibrinogen was detected predominantly on/in macrophages in inflammatory demyelinating areas (figures 1 and 2). Similar to C1q and C3d, macrophage immunostaining for IgG and fibrinogen was low in all other areas (for both IgG and fibrinogen, inflammatory demyelinating areas vs. any other area $p<0.001$).

Macrophage associated immunopositivity for the different complement proteins was highly correlated ($p<0.01$) and immunopositivity for all complement activation products was highly correlated with fibrinogen and IgG ($p<0.01$).

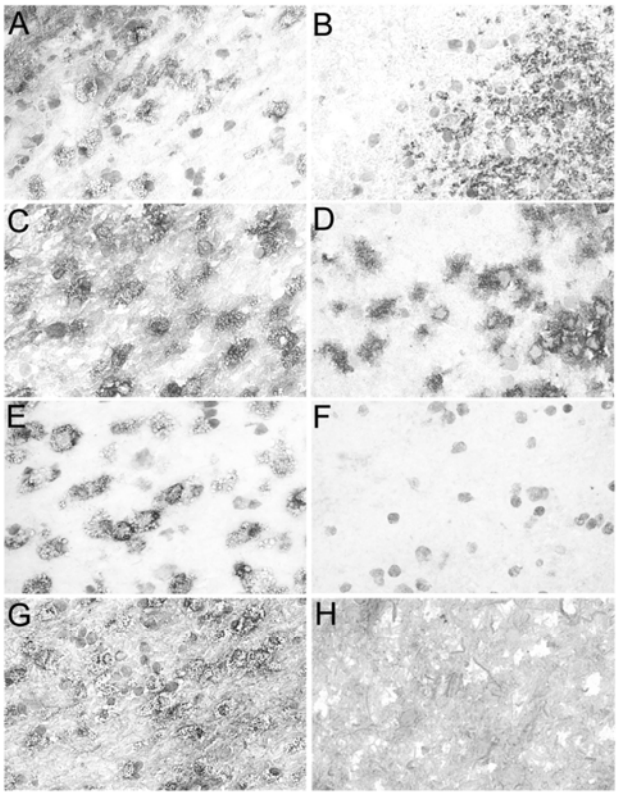

Figure 1.
Complement activation products and IgG are present on and within macrophages in inflammatory demyelinating lesion areas. Left panel: inflammatory demyelinating area; right panel: inflammatory non-demyelinating area. Both areas were located at the border of a chronic active MS lesion. Macrophages in inflammatory demyelinating areas contain intracellular PLP (A) whereas macrophages in inflammatory non-demyelinating areas do not (B). Macrophages in both inflammatory demyelinating and non-demyelinating areas express high levels of HLA-DR (C, D). Expression of C5b-9 is associated with macrophages in inflammatory demyelinating areas (E) but not inflammatory non-demyelinating areas (F). Diffuse immunostaining for IgG, and IgG staining associated with astrocyte processes is observed in demyelinating (G) and non-demyelinating (H) lesion areas, but staining on and within macrophages is restricted inflammatory demyelinating areas (G).
See page 163 for a full-colour representation of this figure.

There was interlesional variation in the extent of macrophage immunopositivity for individual complement activation markers. This variation was intraindividual rather than interindividual. Importantly, increased macrophage immunostaining for at least one, but in most cases more than one (>90% of lesions), of the complement markers was observed in all active demyelinating lesions. There was thus no interindividual heterogeneity observed with respect to macrophage associated complement immunoreactivity.

Complement deposition is occasionally observed on myelin sheaths

C3d immunostaining was occasionally detected on myelin sheaths in inflammatory demyelinating areas (figure 3A,B). C3d myelin staining was infrequent in inflammatory non-demyelinating areas and very rare in inactive lesion areas. No C3d was detected on myelin in NAWM or

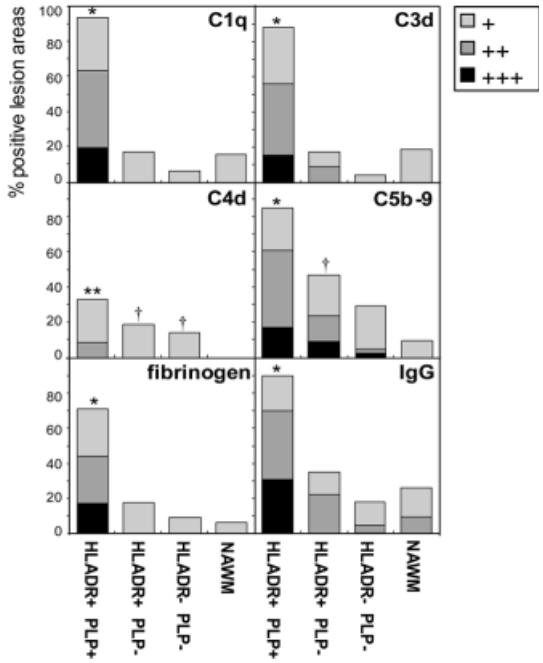

Figure 2.

Semi-quantitative analysis of macrophage associated immunostaining for complement activation products, IgG and fibrinogen in lesions areas of different demyelinating and inflammatory activity. The percentage of areas that was positive for the plasma proteins is represented by the height of the bars, whereas the shading of the bars represents the extent of immunopositivity. (+) light immunopositivity, (++) moderate immunopositivity and (+++) strong immunopositivity.

*immunostaining significantly higher than in all other areas (C1q, C3d, fibrinogen and IgG: $p<0.001$; C5b-9: $p<0.01$); **immunostaining higher than inactive and NAWM areas ($p<0.05$); †immunostaining higher than NAWM ($p<0.01$).

control white matter tissue (figure 3D). C4d staining on myelin sheaths was observed in inflammatory areas with and without active demyelination (figure 3C,D), while staining was lower in inactive lesion areas. Infrequent myelin immunopositivity for C4d was also observed in NAWM and control tissue (figure 3D). Myelin sheaths that were immunopositive for C3d or C4d frequently had an irregular, vacuolated or swollen morphology. Occasionally complement immunopositive myelin sheaths were in direct contact with complement positive macrophages. In these areas increased complement immunopositivity could be observed at the myelin/ macrophage interface (figure 3B). Complement factors C1q, C5b-9, IgG and fibrinogen were not observed on myelin sheaths in any lesion area, NAWM or control tissue.

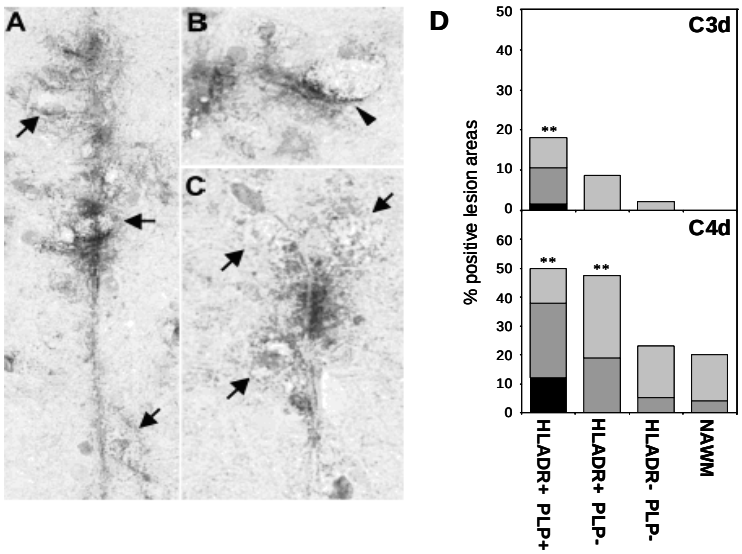

Figure 3.

C3d and C4d are detected on myelin sheaths in inflammatory demyelinating areas. C3d immunostaining is observed on a myelin sheath that is in close contact with macrophages (A, macrophages indicated by arrows), immunostaining is more intense at the interface of the myelin sheath and the macrophage (B, interface indicated by arrowhead). C4d immunostaining on a myelin sheath that is surrounded by macrophages (C, macrophages indicated by arrows). Original magnification 400X. (D) Results of semi-quantitative analysis of myelin associated immunostaining for C3d and C4d. The percentage of areas that were positive for complement is represented by the height of the bars, whereas the shading of the bars represents the extent of immunopositivity.(+) light immunopositivity, (++) moderate immunopositivity and (+++) strong immunopositivity.

**immunostaining higher than in inactive lesions and NAWM ($p<0.05$).

See page 166 for a full-colour representation of figure 3A-C.

Astrocytes

Astrocytes in all lesion areas were immunopositive for C1q, C3d, IgG, fibrinogen and to a lesser extent C4d (data not shown). The cellular pattern of complement immunostaining was different from that of macrophages, with a diffuse staining throughout the astrocyte cytoplasm and processes. No astrocyte immunopositivity was observed for the C5b-9 complex. Surprisingly, astrocyte immunostaining for C1q and IgG was more intense in inflammatory non-demyelinating lesions than in inflammatory demyelinating areas ($p<0.01$). For all other complement proteins, astrocyte immunopositivity was comparable in lesion areas of different inflammatory and demyelinating activity, and generally slightly enhanced over NAWM. In control white matter, astrocyte immunopositivity for complement was rare. Astrocyte associated C1q, C3d, IgG and fibrinogen immunostaining was significantly lower in control white matter than in any lesion area or NAWM ($p<0.05$) (data not shown).

Blood vessels

Immunostaining for C1q, C3d, C4d, IgG and fibrinogen was observed in blood vessel walls on the luminal and abluminal sides of endothelial cells, with no increased immunopositivity associated with MS lesions.

Immunopositivity for IgG, but not complement components, was observed on astrocytic endfeet. The extent of immunostaining for C5b-9 in blood vessel walls was lower than for all complement and plasma proteins studied (data not shown).

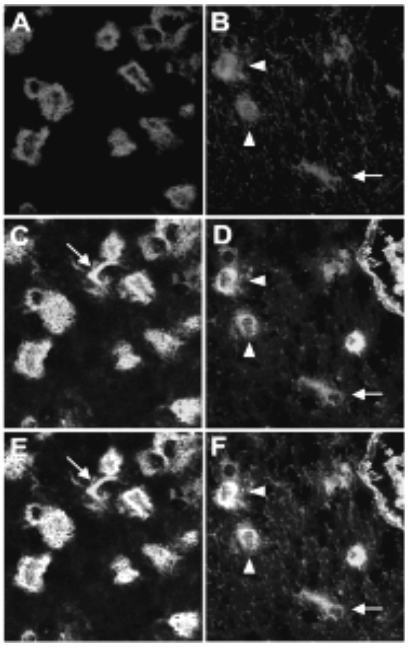

Figure 4.

Double labelling immunofluorescence reveals colocalisation of PLP with complement and complement with IgG in inflammatory demyelinating areas. The left panel shows colocalisation of PLP (A) and C3d (C) on the surface and in vesicle-like structures inside phagocytic macrophages (E shows merge of A and C). Immunostaining for C3d, but not PLP, is observed on astrocytes (arrow). The right panel shows colocalisation of C1q (B) with IgG (D) on infiltrating phagocytic monocytes/macrophages (arrowheads) and on a glial cell (arrow) (F shows merge of B and D). Vascular immunostaining for IgG, but not C1q, is observed on the inner and outer basement membranes lining the Virchow-Robin space (D, F, asterix). The IgG-positive, C1q-negative cell on the left side of image D and F may represent a plasma cell. Original magnification 400X.

See page 167 for a full-colour representation of this image.

Colocalisation of complement with PLP and IgG in phagocytic macrophages

The presence of complement activation products, IgG, and myelin in vesicular structures within macrophages in active demyelinating areas suggests a role for complement and IgG in myelin phagocytosis. We next investigated if these proteins were present simultaneously within the same cells, or in the same structures within the cells. Double labelling studies demonstrated that PLP and C3d colocalised in structures resembling phagocytic vesicles within macrophages (figure 4A,C,E). Similarly, C1q and C4d colocalised with PLP in phagocytic macrophages (data not shown). In addition, double fluorescent staining for C1q and IgG demonstrated colocalisation of complement and IgG in macrophages in inflammatory demyelinating areas (figure 4B,D,F).

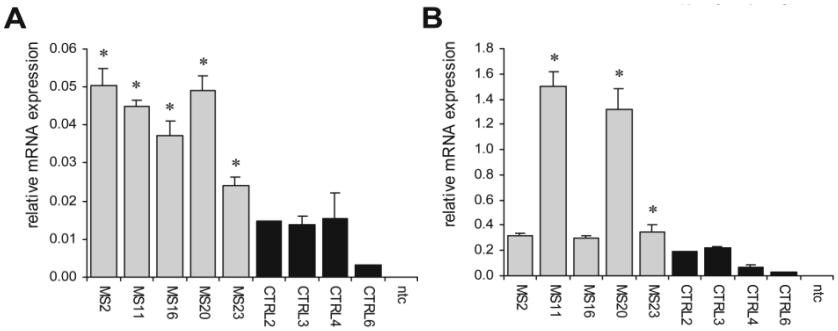

Figure 5.

Enhanced local expression of complement mRNA in tissue blocks containing inflammatory demyelinating areas. Figure represents relative expression of C1q and C3 calculated using expression of cyclophilin A as a reference gene. (A) Expression of C1q mRNA was enhanced in 5/5 MS tissue blocks (shaded bars) when compared to control white matter tissue blocks (black bars)(*, $p<0.05$). (B) Expression of C3 mRNA was enhanced in 3/5 MS tissue blocks (shaded bars) when compared to control white matter tissue blocks (black bars)(*, $p<0.05$).

Enhanced local production of complement in inflammatory demyelinating lesion areas

The presence of C1q and C3d on and within macrophages in inflammatory demyelinating areas strongly correlated with deposition of fibrinogen and IgG, suggesting that complement proteins had entered the brain by leakage through the blood brain barrier. However, resident cells of the CNS as well as infiltrating macrophages are able to produce complement proteins (Veerhuis *et al.*, 1996; Yasojima *et al.*, 1999). To examine whether local production of complement plays a role in MS lesion formation, expression of C1q and C3 mRNA was determined in MS tissue blocks containing inflammatory demyelinating areas from five MS patients and normal white matter tissue blocks from four non-neurological controls. Relative abundance of C1q and C3

mRNA was calculated using the expression levels of two genes, cyclophilin A and GAPDH, as an internal reference. The results obtained with the two different reference genes were highly comparable. C1q mRNA expression was increased in all MS tissue blocks when compared to the average expression in control tissue ($p<0.05$; figure 5A). Expression of C3 mRNA was increased as well, although less consistently. Expression of C3 was significantly enhanced in

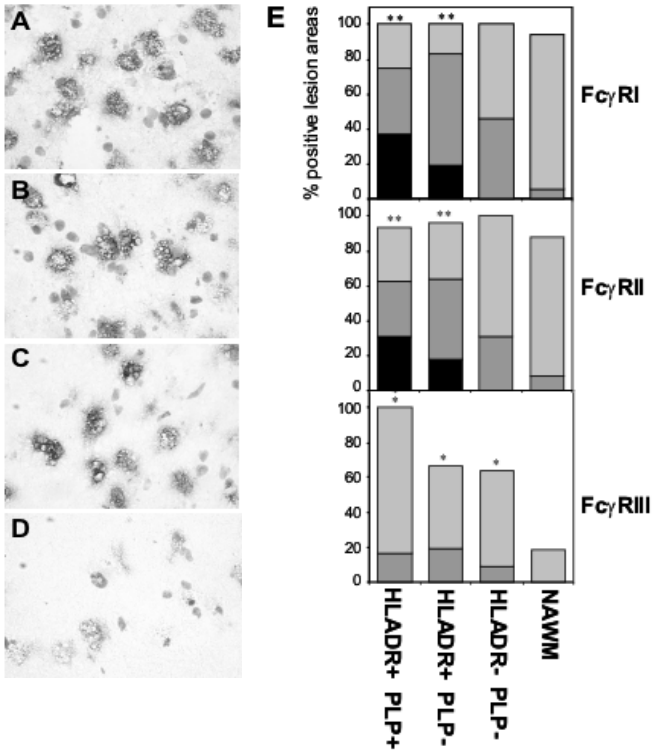

Figure 6.

High expression of Fcγ R in inflammatory a ession of HLA-DR (A) on phagocytic macrophages in an inflammatory demyelinating area at the border of a chronic active lesion. In the same area, high expression of Fcγ RI (B) and Fcγ RII (C) is detected on the surface of phagocytic macrophages and within intracellular vesicle-like structures. Expression of Fcγ RIII (D) is observed in association with phagocytic macrophages as well, although the extent of immunostaining is lower than for Fcγ RI and Fcγ RII. Original magnification 400X. (E) Results of semi-quantitative analysis of Fcγ R in different lesion areas. The percentage of lesion areas that were positive for Fcγ R expression is represented by the height of the bars, whereas the shading of the bars represents the extent of immunopositivity. (+) light immunopositivity, (++) moderate immunopositivity and (+++) strong immunopositivity.

**immunostaining higher than in inactive lesions and NAWM ($p<0.05$), *immunostaining higher than NAWM ($p<0.05$).

See page 170 for a full-colour representation of figure 4A-D.

three out of five MS patients ($p<0.05$; figure 5B). This demonstrates that at least part of the complement products in areas of active demyelination is produced locally.

Expression of Fcγ receptors in MS lesion areas is associated with inflammation

Macrophage immunostaining for FcγRI and FcγRII was consistently enhanced in inflammatory lesion areas, both demyelinating and non-demyelinating (figure 6). Expression of FcγRI and FcγRII was lower in inactive lesions areas and NAWM ($p<0.05$). Macrophage expression of FcγRIII was similar in all lesion areas, and in all cases enhanced over NAWM ($p<0.05$) (figure 6). The results are summarized in figure 6E.

In inflammatory demyelinating areas, staining for all FcγRs was observed in intracellular vesicle-like structures in phagocytic macrophages and on the cell surface, similar to immunostaining for HLA-DR (6A-D). In these areas, double-labelling studies revealed colocalisation of FcγRI and PLP within macrophages (data not shown). Furthermore, Fcγ receptors colocalised with IgG and C1q (figure 7).

Constitutive expression of FcγRI, FcγRII and to a lesser extent FcγRIII was observed on microglia and perivascular macrophages in NAWM areas of MS patients and in normal controls. Strikingly, expression of FcγRII, but not FcγRI or FcγRIII was particularly high on perivascular macrophages, both in lesion and control areas (data not shown). FcγRIII was the only FcγR that was expressed on endothelial cells. Expression was restricted to capillaries and small venules, and unchanged in lesion areas when compared to NAWM (data not shown).

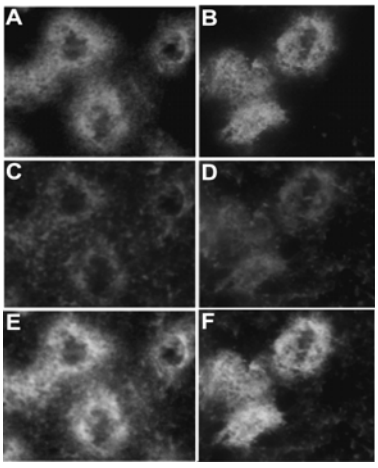

Figure 7.

Double labelling immunofluorescence reveals colocalisation of FcγR with IgG and complement an inflammatory demyelinating area at the border of a chronic active MS lesion. The upper panel shows colocalisation of FcγRII (A) and IgG (C) on the surface and in vesicle-like structures inside phagocytic macrophages (E shows merge of A and C). The lower panel shows colocalisation of FcγRII (B) with C1q (D) on and within phagocytic macrophages (F shows merge of B and D). Diffuse background staining for IgG (C) and C1q (D) is typical of inflammatory demyelinating areas in the CNS. Original magnification 400X.

See page 171 for a full-colour representation of this image.

Discussion

This study demonstrates that complement activation products, IgG and FcγR are consistently present on and within phagocytic macrophages in active demyelinating white matter lesions in MS patients. Presence of complement and IgG was mostly restricted to inflammatory demyelinating areas, whereas enhanced expression of FcγR was observed in all inflammatory areas, both demyelinating and non-demyelinating. Furthermore, local production of complement products was enhanced in active demyelinating MS lesions. This suggests an important role for complement, IgG and FcγR in white matter demyelination in MS.

MS lesions containing inflammatory demyelinating areas are rare in CNS autopsy material from patients with established MS. This may explain why previous studies focusing on active demyelinating lesions were either restricted to a few patients (Storch *et al.*, 1998; Bruck *et al.*, 2001; Prineas *et al.*, 2001) or performed using brain material from early MS patients, often with acute disease (Gay *et al.*, 1997; Lucchinetti *et al.*, 2000). Analysis of a large number of active demyelinating MS lesions by Lucchinetti *et al*. (2000) suggested that these lesions can be subdivided into four immunopathological patterns. MS lesions patterns varied between patients, but not within patients, implicating heterogeneity of MS pathogenesis. One pattern of demyelination (pattern II) was characterized by presence of immunoglobulins and complement activation product in association with macrophages containing intracellular myelin debris. This pattern was observed in approximately fifty percent of MS patients with acute MS, but appeared to be more prominent in patients with disease duration longer than one year (Lucchinetti *et al.*, 2000).

The data presented here show complement activation products and IgG within PLP-positive macrophages in all active demyelinating lesions in all patients that were included. This indicates that the distribution of MS lesion patterns in an unselected material of MS patients with a typical disease course is different from that of patients with very severe disease or a tumour like presentation.

In a recent study, oligodendrocyte apoptosis and complement deposition on myelin sheaths were proposed to be the earliest events in the formation of new lesions in acute MS (Barnett and Prineas, 2004), while similar oligodendrocyte changes were not observed in regions of active demyelination in established lesions of relapsing remitting or chronic MS (Prineas *et al.*, 2001; Barnett and Prineas, 2004). Different pathways of demyelination may converge with time resulting in a common pathway of demyelination in later phases of disease. Furthermore, some of the immunopathological patterns as described by Lucchinetti *et al*. (2000) may be restricted to patients with acute forms of MS that will never reach the chronic phase of disease.

Elements of the different pathological subtypes may exist within one lesion. This was recently described for highly acute, newly forming MS lesions, where complement activation was observed in areas of oligodendrocyte apoptosis (Barnett and Prineas, 2004). Whether this is the case in the present material is not known, as the presence of oligodendrocyte apoptosis and preferential loss of myelin associated glycoprotein was not studied. The extent of oligodendrocyte apoptosis in this patient material is subject of further studies in our laboratory.

Although complement activation products and IgG were consistently detected together with myelin on and within macrophages in areas of active demyelination, myelin staining for C1q, C5b-9 and IgG was largely absent, and C3d and C4d were only occasionally found on myelin sheaths. Deposition of C3d on myelin in absence of full complement activation (i.e. presence of C5b-9) has been described before (Prineas *et al.*, 2001), although other studies reported C9neo immunopositivity on myelin in areas of active demyelination (Storch *et al.*, 1998; Lucchinetti *et al.*, 2000; Barnett and Prineas, 2004). C3d and C4d covalently bind to their target after cleavage and may thus accumulate at the site of deposition, whereas non-covalently bound C1q and C5b-9 are rapidly turned over, by detachment or vesiculation respectively (Morgan, 1989). *In vitro* studies demonstrated the capacity of oligodendrocytes to secrete vesicles bearing the C5b-9 complex, possibly explaining the absence of immunopositivity for C5b-9 on myelin sheaths (Scolding *et al.*, 1989).

In cortical lesions, deposition of C4d on myelin was observed in absence of other complement activation products on myelin or other structures (Schwab and McGeer, 2002; Brink *et al.*, 2005). However, active demyelinating white matter lesions in the same MS tissues showed immunopositivity for complement activation products on macrophages, astrocytes and myelin (Brink *et al.*, 2005). This is consistent with location dependent differences in lesion pathology, as cortical lesions have been found to be largely non-inflammatory (Peterson *et al.*, 2001).

Not much is known on the origin of complement proteins in MS lesions. Enhanced expression of mRNAs for C1q and to a lesser extent C3 in MS lesions demonstrates that at least part of the complement proteins in areas of active demyelination are produced locally. Constitutive expression of C1q in the CNS is substantially lower than expression of C3 (Yasojima *et al.*, 1999), possibly explaining the difference in regulation under inflammatory conditions. Similarly, in Alzheimer's disease, upregulation of C1q mRNA in the CNS was more obvious than upregulation of C3 transcription (Yasojima *et al.*, 1999). Gene-microarray analysis of active MS lesions previously showed enhanced mRNA expression for C1r (Lock *et al.*, 2002), a protein that forms the C1 complex with C1q and C1s. It is unknown what cell type is responsible for the upregulation of C1q and C3. Astrocytes in all lesion areas were immunopositive for complement products, while immunopositivity associated with macrophages was increased in inflammatory demyelinating areas, suggesting that macrophages are responsible for the enhanced local production of C1q and C3. In CNS derived primary cultures, microglia were the only cells that produced C1q and production was enhanced under inflammatory conditions. C3 was produced by astrocytes, microglia and neuroblastoma cell lines (Veerhuis *et al.*, 1999). Further studies using in situ hybridisation techniques are required to elucidate patterns of local complement expression in MS lesions.

The colocalisation of PLP and IgG in phagocytic macrophages (this study), and the association of anti-MOG antibodies with degraded myelin within phagocytic macrophages (Genain *et al.*, 1999), suggest a role for antibody-mediated uptake of myelin, possibly through Fcγ receptor mediated phagocytosis. Although enhanced expression of FcγR on microglia and infiltrating macrophages in active MS lesions was demonstrated before (Ulvestad *et al.*, 1994) it was unknown if FcγR colocalised with IgG and complement in areas of active demyelination. The present study demonstrates colocalisation of FcγR with IgG, complement and PLP in vesicle

like structures in phagocytic macrophages, suggesting a role of FcγR mediated myelin phagocytosis in MS. Crosslinking of FcγR is achieved most efficiently by immune complexes (IC). Indeed, PLP, C1q, IgG and FcγR colocalised within macrophages in inflammatory demyelinating areas, suggesting a role for FcγR mediated uptake of IC containing myelin antigens in MS. Crosslinking of macrophage FcγR can induce a wide variety of effector functions, including phagocytosis, release of inflammatory mediators, antigen presentation (Ravetch and Bolland, 2001), all of which may contribute to MS lesion formation. The result of FcγR crosslinking depends on the balance between activating and inhibitory FcγR that are expressed on the cell surface (Takai, 2002). In this respect, it would be very interesting to separately analyse expression of the activating and inhibitory subclass of FcγRII (FcγRIIa and FcγRIIb respectively). Unfortunately, the available antibodies do not distinguish between FcγRIIa and FcγRIIb. Expression of the activating FcγRI, but not FcγRII, is enhanced in the presence of IFN-γ. As IFN-γ production is enhanced in MS (Sarchielli *et al.*, 1997), the expression pattern of FcγR on macrophages in MS lesions may be skewed towards the activating FcγR, implicating that FcγR crosslinking will have a pro-inflammatory effect. In support of this, microglia were shown to produce inflammatory chemokines upon *in vitro* FcγR crosslinking under pro-inflammatory conditions (Song *et al.*, 2002).

In summary, deposition of complement and IgG was consistently observed on and within macrophages in inflammatory demyelinating MS lesion areas, where local production of complement was enhanced. Furthermore, complement activation products, IgG and FcγR colocalised with intracellular PLP in phagocytic macrophages. It is concluded that all tools for antibody- and complement-mediated phagocytosis are present in areas of active demyelination. Consistent presence of complement and IgG in areas of active demyelination demonstrates that the distribution of lesion patterns in an unselected MS autopsy material from patients with typical MS disease course is different from the patterns that were previously described in a population of patients with early MS.

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Chapter 6

Myelin flow cytometry assay: a new method to detect antibodies directed against human myelin

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Abstract

Antibodies are thought to contribute to CNS inflammation and demyelination in a subgroup of multiple sclerosis (MS) patients. However, the antigen specificity of antibodies in MS is unknown. Antibodies directed against a number of myelin proteins have been detected in serum of multiple sclerosis (MS) patients, but no single antigen has been associated exclusively with MS.

Studies in animal models suggest that antibodies with demyelinating capacity are directed against native conformational and post-translationally modified epitopes. The current methods to detect anti-myelin antibodies often do not allow recognition of such epitopes, either because recombinant proteins or peptides are used as antigens or because the techniques that are used require denaturation of protein antigens. This is particularly relevant for myelin oligodendrocyte protein (MOG), the myelin protein that has been shown to elicit potent demyelinating antibodies *in vivo*.

We have developed a reproducible, flow cytometry-based assay to detect serum antibodies directed against human whole myelin, including antibodies to myelin basic protein, proteolipid protein and MOG. Myelin was isolated from human white matter CNS tissue, allowing antibody recognition of conformational and post-translationally modified epitopes.

Using this assay, anti-myelin antibodies were measured in serum of 56 MS patients and 27 healthy donors (HD). Approximately fifty percent of MS patients showed enhanced anti-myelin IgG levels when compared to HD. This suggests that antibody responses to native human myelin antigens may be relevant in MS, and that antibodies to human whole myelin may be a valuable biomarker to identify patients with antibody-mediated inflammation.

Introduction

Abnormalities in the humoral immune system are a common observation in Multiple Sclerosis (MS). Serum and cerebrospinal fluid (CSF) from MS patients have been screened for antibodies directed against a wide range of myelin antigens, including myelin basic protein (MBP), proteolipid protein (PLP), myelin associated glycoprotein (MAG), oligodendrocyte specific protein (OSP) and myelin oligodendrocyte glycoprotein (MOG) (Sellebjerg *et al.*, 1998; Bronstein *et al.*, 1999; Reindl *et al.*, 1999; Lindert *et al.*, 1999; Vojdani *et al.*, 2003; Berger *et al.*, 2003). Although enhanced anti-myelin antibody responses in MS patients have been described, the results of these studies are inconsistent, and thus far no single antigen has exclusively been associated with MS.

This inconsistency is possibly related to heterogeneity in pathogenic processes underlying MS, but also to the variety of different assays that are currently used to identify anti-myelin antibodies. In addition, the source of myelin proteins that are used for screening is highly variable. Screening studies are often performed using recombinant myelin proteins that are expressed in bacteria, mammalian cells or by *in vitro* translation, all of which differently affect post-translational processing such as protein folding and glycosylation, further diversifying the results. This is particularly relevant for the minor myelin proteins (MOG and MAG) that are difficult to isolate from the CNS in sufficient quantities, although recombinant proteins and peptides have also been used to screen for antibodies directed against the major myelin proteins MBP and PLP (Vojdani *et al.*, 2003).

MOG has been a favourite target in anti-myelin antibody screening studies. MOG is located in the outermost layer of the intact myelin sheaths, where it is easy accessible for autoimmune responses (Baumann and Pham-Dinh, 2001). The relevance of MOG as an auto-antigen has been demonstrated in experimental allergic encephalomyelitis (EAE), where immunization with MOG can induce chronic demyelinating disease with CNS lesions highly reminiscent of MS, associated with a MOG specific T- and B-cell response (Adelmann *et al.*, 1995; Brok *et al.*, 2000). The *in vivo* demyelinating potential of anti-MOG antibodies has repeatedly been demonstrated in models of antibody-exacerbated EAE (Linington *et al.*, 1988; Morris-Downes *et al.*, 2002). Importantly, pathogenic anti-MOG antibodies were directed against conformational epitopes, and it was shown that these conformational antibodies not only enhanced demyelination but also contributed to dissemination of demyelinating lesions in the CNS (Brehm *et al.*, 1999; von Budingen *et al.*, 2004).

The methods that are currently used to detect anti-MOG antibodies often do not allow recognition of conformational epitopes, suggesting that the pathogenically relevant anti-MOG responses may be ignored. Similarly, antibodies specific to other myelin antigens may be directed against conformational or posttranslationally modified epitopes. In addition, the anti-myelin antibody response in MS patients may be directed against a range of myelin antigens rather than to one particular protein, due to the continuous release of myelin antigens during chronic CNS demyelination and inflammation in MS.

We here describe an assay to detect serum antibodies directed against human whole myelin. This assay enables detection of antibodies directed against native, posttranslationally modified

antigens of a wide range of myelin proteins, including MOG. Taking advantage of the bead-like appearance of myelin in aqueous solution, we analysed binding of human serum immunoglobulins to whole myelin using flow cytometry. Using the myelin flow cytometry assay, serum samples of MS patients and healthy donors were screened for the presence of anti-myelin antibodies.

Materials and Methods

Isolation of human myelin

Human CNS white matter tissue was obtained at autopsy from individuals without a history of neurological disease, in collaboration with the Dutch Brain Bank (coordinator: Rivka Ravid). Myelin was isolated according to the method of Norton and Poduslo (Norton and Poduslo, 1973), with minor adaptations. Briefly, CNS white matter was homogenised in 0.32 M sucrose. The suspension was layered over a 0.85 M solution of sucrose and centrifuged at 75,000 g for 30 min at 4°C (step 1). The interphase, containing the myelin, was pooled and washed three times with de-ionised water by repeated centrifugation at 75,000 g for 15 min at 4°C (step 2). Steps 1 and 2 were repeated twice. Myelin was collected and stored at –20°C until further use.

The total protein concentration of isolated myelin was calculated using a bovine serum albumin (BSA) standard curve as described (Van der Goes *et al.*, 1999). Myelin from five subjects was pooled for use in all assays.

Myelin Flow Cytometry Assay

Human myelin (15 μg) and undiluted serum (4 μl) were added to 100 μl PBS in a 96-well V-bottom plate and incubated for 30 min at 37°C or overnight at 4°C. Unbound serum proteins were removed by washing the myelin in PBS (4,500 rpm, 4 min, 3 times repeated). Myelin was subsequently incubated with polyclonal rabbit-anti-human Ig (detecting a combination of human IgG, IgM and IgA; DAKO, Glostrup, Denmark) for 30 min at 37°C. After washing in PBS, samples were incubated with Phycoerythrin (PE)-labelled donkey-anti-rabbit Ig (Jackson Laboratories, West Grove, PA, US), washed again and taken up in 50 μl PBS in FACS tubes (B&D Biosciences, Franklin Lakes NJ, US). Alternatively, myelin was incubated with biotinylated goat-anti-human IgG or biotinylated donkey-anti-human IgM (Jackson Laboratories) to specifically detect IgG or IgM bound to myelin. After washing, samples were incubated with Alexa$^{488/594}$-conjugated streptavidin (Molecular Probes, Eugene, OR, US), washed and taken up in 50 μl PBS in FACS tubes.

As a positive control, human myelin was incubated with 2 μg/μl monoclonal antibodies directed against MOG (Z12 mAb, kindly provided by Dr. Piddlesden (Piddlesden *et al.*, 1993) and 8-8C15 (Linnington *et al.*, 1984), kindly provided by Dr. Reindl), myelin basic protein (MBP; clone MBP22 (Groome *et al.*, 1988), kindly provided by Dr. Groome) and proteolipid protein (PLP; clone plpc1, Serotec, Oxford, UK). Binding of primary antibodies was detected using PE-labelled rabbit anti-mouse Ig (Jackson Laboratories). As negative controls, myelin was incubated with primary mouse antibodies of irrelevant specificity (ED1, Serotec, Oxford, UK) or PBS before incubation with conjugate antibody.

Myelin immunoreactivity was measured using the FACS Calibur (B&D Biosciences, Franklin Lakes NJ, US). Anti-myelin antibody responses were expressed as the mean fluorescence intensity (MFI) of 20,000 events measured by the flow cytometer. All serum samples were incubated with myelin in triplicate and all samples were included in at least three separate experiments.

Patient characteristics

Serum was obtained from 27 healthy donors (HD) and 56 MS patients. In 19 patients disease course was relapsing remitting (RRMS), in 18 patients secondary progressive MS (SPMS) and 19 patients primary progressive (PPMS). The median age of MS patients was 48 years (range 28–65); the median age of HD was 37 years (range 29–62) (significantly lower than MS patients, $p<0.01$). The median age of RRMS patients was 43 years (range 28-56). The median age of SPMS patients was 47 years (range 33-57; higher than HD, $p<0.05$) and the median age of PPMS patients was 53 years (range 29-62; higher than RRMS, SPMS and HD, $p<0.05$). Of the MS patients 39/51 were female (16/18 of RRMS patients, 9/14 of SPMS patients and 10/19 of SPMS patients) of the HD 15/27 were female (gender distribution in MS and HD not significantly different).

The study was performed in accordance with the Helsinki Declaration and with approval of the local ethical committee.

Statistical analysis

It was technically not feasible to test all MS and HD samples in one experiment. Nevertheless, correlations between MFI values of serum samples that were included in consecutive experiments were high (Pearson's Rho: 0.6-0.8, $p<0.001$). Therefore, MFI values from two representative experiments were normalised and pooled for further analysis. For normalisation, regression analysis was performed on samples that were included in both experiments and the regression line was used to normalize MFI values.

Differences in median age between groups (MS patients, MS clinical subgroups and HD) were analysed using Mann-Whitney U-test. Difference in distribution of females and males in MS patients and HD were analysed using Chi-square test. Differences in anti-myelin antibody response between MS patients and HD, or between clinical subgroups were calculated using the Mann-Whitney U test. Anti-myelin antibody levels in individual MS serum samples were considered to be enhanced over HD values when the MFI was higher than the [mean value of HD + 2.5*standard deviation]. Possible differences in the proportion of anti-myelin Ig positive samples between clinical subgroups of MS patients were analysed using the Chi-square test. P values lower than 0.05 were considered to indicate statistical significance (confidence level 95%).

Results

Human myelin flow cytometry assay detects monoclonal antibodies directed against myelin proteins

Incubation of human myelin with a murine monoclonal anti-MOG antibody (8-18C5), but not with an isotype control antibody (ED1), resulted in a shift in mean fluorescence intensity (MFI) as shown in Figure 1A. In addition, myelin was recognized by well-characterised murine monoclonal antibodies directed against MBP, PLP and an additional anti-MOG antibody (Figure 1B). No binding was detected using an antibody of irrelevant specificity or when the primary antibody was substituted by PBS. This demonstrates that the human myelin flow cytometry assay specifically detects antibodies directed against myelin proteins.

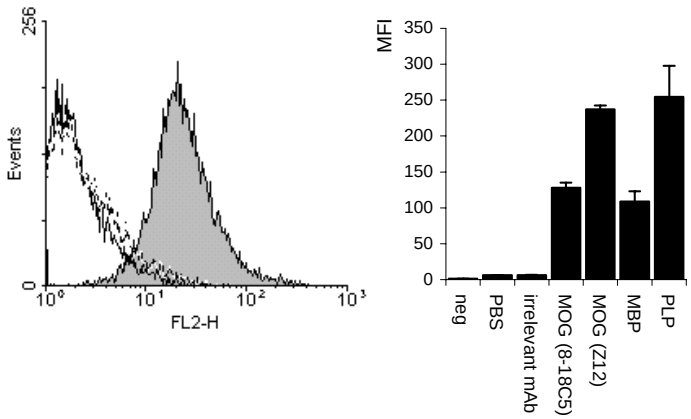

Figure 1.
Human myelin binds well-characterised monoclonal anti-myelin antibodies. (A) Representative plot showing a shift in MFI after binding of monoclonal anti-MOG 8-18C5 to human myelin (thin line: conjugate only; dashed line: isotype ctrl (ED1); filled histogram: anti-MOG 8-18C5). (B) Binding of antibodies directed against MOG, MBP and PLP to human myelin.

Human myelin binds serum antibodies from MS patients and healthy donors (HD)

To detect anti-myelin antibodies in serum of MS patients and HD, the myelin flow cytometry assay was performed using human serum as a source of primary antibody. Flow cytometry plots for an HD (HD7, with MFI representative for average value HD), and an MS patient (MS12) showing enhanced anti-myelin Ig binding to whole human myelin are shown Figure 2. Separate analysis of anti-myelin IgG and IgM demonstrates that binding of anti-myelin IgG was higher for MS12 than for HD7, whereas anti-myelin IgM levels were comparable.

Each serum sample was assayed in at least three different experiments. The correlation between MFI values obtained in separate experiments was high (Pearson's Rho: 0.6-0.8 $p<0.001$), demonstrating that the assay was reproducible.

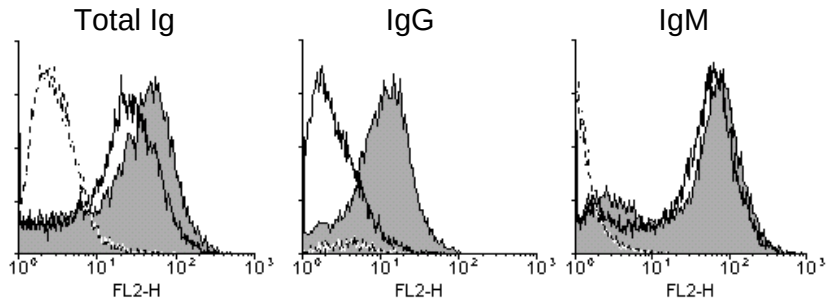

Figure 2.
Myelin flow cytometry assay detects anti-myelin antibodies in human serum. Representative flow cytometry plots showing a shift in MFI after binding of serum Ig, IgG and IgM from an MS patient (MS12, filled histogram) and a healthy donor (HD7, open histogram, solid line) to human whole myelin. The dashed line represents the conjugate control. MS12 shows slightly enhanced binding of total Ig when compared to HD7 (left panel). This can be attributed to enhanced binding of anti-myelin IgG (middle panel) but not anti-myelin IgM (right panel).

At the group level, anti-myelin antibody levels (total Ig) were slightly higher in MS patients than in HD, although considerable overlap existed. The difference did not reach the level of significance ($p<0.063$; Figure 3). In contrast, separate analysis of IgG responses showed that anti-myelin IgG in MS patients was significantly enhanced over HD ($p<0.001$). Anti-myelin IgM responses were heterogeneous in both MS patients and healthy donors, there was no significant difference between the groups.

Analysis of anti-myelin total Ig levels in clinical subgroups of MS patients revealed that SPMS patients showed the highest response; this was the only clinical subgroup that showed significantly enhanced MFI values compared to HD ($p<0.05$). The differences between anti-myelin Ig responses in clinical subgroups of MS were not significant.

RRMS, SPMS and PPMS patients all showed significantly enhanced anti-myelin IgG when compared to HD ($p<0.001$ for all subgroups vs. HD), with no significant differences between the clinical subgroups. IgM responses were comparable in all clinical subgroups and HD.

As MS is a heterogeneous disease, anti-myelin antibody responses may be very different among patients. It may thus be more relevant to compare individual MS patients to a control population than to look at the MS population as a whole. Individual anti-myelin antibody levels in MS patient sera were compared to the average level of HD serum. Cut-off values [average HD+2.5SD] for Ig, IgG and IgM are indicated in Figure 3. In 2/56 MS patients anti-myelin Ig levels were higher than in HD, while all HD showed MFI values below the cut-off value. Anti-myelin IgG levels higher than the cut-off value were observed in 24/51 MS patients

but in none of the HD. Enhanced anti-myelin IgM levels were detected in 2/50 MS patients, however 1/24 HD also showed enhanced IgM, emphasising the heterogeneity of the IgM response (Table 1). The distribution of anti-myelin antibody positive serum samples was not significantly different between clinical subgroups (Table 1).

Table 1. MS patients and healthy donors showing enhanced anti-myelin antibody responses

	Total Ig	IgG	IgM
MS patients	2/56	24/51	2/50
RRMS	0/19	9/18	0/18
SPMS	2/18	9/14	2/14
PPMS	0/19	6/19	0/18
Healthy donors	0/27	0/24	1/24

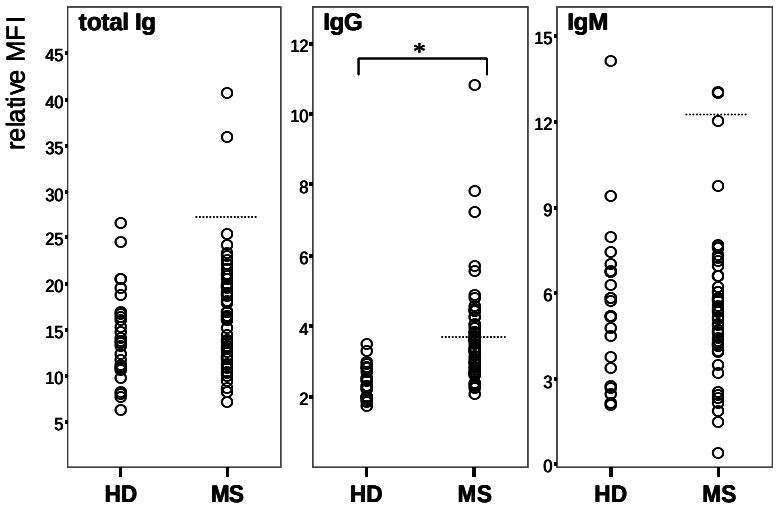

Figure 3.

Anti-myelin antibody response in MS patients and HD. Left panel shows slightly elevated anti-myelin Ig levels in MS patients and the middle panel shows significantly enhanced anti-myelin IgG in MS patients (*p<0.001, Mann-Whitney U test). The right panel shows heterogeneous anti-myelin IgM responses in MS patients as well as HD. Dotted lines indicate the [average MFI value HD+2.5*SD], representing the cut-off value for 'anti-myelin antibody positivity'. Data points above the dotted lines represent MS patients (or HD) with elevated anti-myelin serum antibody responses.

Discussion

We present a reproducible assay to detect anti-myelin antibodies in serum of MS patients. The assay was developed to detect antibody responses against human whole myelin, rather than against individual myelin proteins. Myelin was isolated from human CNS white matter, enabling us to detect antibody responses against native myelin proteins, including conformational and posttranslationally modified epitopes. The assay is performed in fluid phase, preventing the possible masking of relevant epitopes due to selective binding of the epitopes to a surface.

Using this assay, we detected enhanced anti-myelin Ig responses in MS patients when compared to healthy donors. Separate analysis of IgG and IgM showed that this difference could be attributed to enhanced anti-myelin IgG in MS patients, whereas anti-myelin IgM was comparable in MS and HD. The anti-myelin antibody response was heterogeneous among MS patients, approximately fifty percent of MS patients showed enhanced anti-myelin IgG. Interestingly, it has been proposed that antibodies contribute to MS lesion formation in a similar proportion of MS patients (Lucchinetti *et al.*, 2000), although it is unknown if such antibody-mediated pathology is reflected in the serum.

Further studies are required to expand the analysis of serum anti-myelin antibodies in larger groups of MS patients, and to correlate anti-myelin antibody responses with other disease parameters such as the expanded disability status scale (EDSS), which was beyond the scope of the current methodological description. Importantly, a pilot study in our laboratory suggested that an antibody responses to whole myelin are also detected in the CSF of MS patients (Van der Goes *et al*., 2004).

Many groups have studied antibody responses against myelin, however in contrast to the present study, analysis was generally restricted to one, two or three individual myelin proteins (Annunziata *et al.*, 1997; Reindl *et al.*, 1999; Lindert *et al.*, 1999; Schmidt *et al.*, 2001; Lutterotti *et al.*, 2002; Vojdani *et al.*, 2003; Lampasona *et al.*, 2004). Although the results have been variable, most studies detected enhanced anti-myelin antibodies in at least a subpopulation of MS patients. This was found most consistently for anti-MOG antibodies (Reindl *et al.*, 1999; Lindert *et al.*, 1999; Schmidt *et al.*, 2001; Vojdani *et al.*, 2003), and to a lesser extent for antibodies directed against MBP (Annunziata *et al.*, 1997; Vojdani *et al.*, 2003).

Whereas results for anti-MBP antibodies were mostly obtained using native protein, results for anti-MOG antibodies were almost exclusively obtained using bacterially expressed recombinant proteins or small peptides derived from the MOG amino acid sequence. In these systems, antibodies directed against conformational and posttranslationally modified epitopes are not detected (Haase *et al.*, 2001). The relevance of antibody responses to such epitopes was demonstrated in experimental allergic encephalomyelitis (EAE). Anti-myelin antibodies that could mediate demyelination *in vivo* were those directed against conformational epitopes, whereas antibodies directed against linear epitopes did not affect the clinical course of EAE (Brehm *et al.*, 1999; von Budingen *et al.*, 2001). Importantly, mouse monoclonal antibodies that showed the highest binding to whole mouse myelin *in vitro* showed the highest

demyelinating capacity *in vivo* (Van der Goes *et al.*, 1999). This suggests that if such antibodies are present in MS patients, they could be detected using the myelin flow cytometry assay that is described in this study.

It was recently shown that MS patients show enhanced levels of serum antibodies directed against native glycosylated mouse MOG, suggesting that conformational epitopes are also relevant in MS. Similar to our results, the difference between healthy donors and MS patients was most obvious for IgG antibodies (Gaertner *et al.*, 2004). It is unknown to what extent the antibody response to whole myelin correlates with the anti-MOG antibody response. This will be subject of further studies in our laboratory.

In summary, the myelin flow cytometry assay is a reliable method to detect and quantify antibody responses directed against human whole myelin. The assay allows detection of antibodies directed against native conformational and posttranslationally modified myelin antigens, including native MOG. The myelin flow cytometry assay will be compared to other assays detecting antibody responses against individual myelin proteins, including MOG and MBP, in a worldwide network. In addition, anti-myelin antibody responses will be compared between MS patients and patients with other neurological diseases. The value of this assay to predict MS disease course is another topic of future research.

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Chapter 7

Myelin flow cytometry assay: clinical relevance of serum anti-myelin antibodies

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Abstract

We recently described a new assay to detect antibodies directed against human whole myelin in MS patients. In contrast to the assays that are currently used to identify anti-myelin antibodies, the new assay allows detection of antibodies directed against conformational and post-translationally modified epitopes.

In the present study, we analysed the clinical relevance of serum anti-myelin antibodies by correlating anti-myelin antibody levels to disease duration and disease severity (measured by EDSS and MSFC). In addition, anti-myelin antibody responses were compared to anti-MOG antibody responses. In almost fifty percent of MS patients (n=99), anti-myelin IgG was enhanced over anti-myelin IgG in HD (n=40), confirming our previous results in another population of MS patients. The anti-myelin IgM response was heterogeneous in both MS patients and HD, although overall anti-myelin antibody levels in MS patients were significantly enhanced. Anti-myelin total Ig levels were comparable in MS patients and HD. Anti-myelin IgG and IgM responses did not correlate with the age of the subjects, MS subtype, disease duration or disease severity. In contrast to anti-myelin IgG, anti-MOG IgG levels were comparable in MS patients and healthy donors and no difference between MS and HD was observed for anti-MOG total Ig or IgM. Nevertheless, total Ig and IgM responses to whole myelin and MOG were significantly correlated. However, no correlation between anti-myelin IgG and anti-MOG IgG was observed. This suggests that the anti-myelin IgG response in MS is directed against a different combination of myelin antigens than the anti-myelin IgM response. As anti-myelin IgG was more specific for MS than IgM, it is possible that anti-myelin IgG antibodies detected in this assay represent pathogenically relevant antibodies.

Introduction

Multiple sclerosis (MS) is a chronic disabling disease of the central nervous system (CNS), neuropathologically characterised by inflammation, demyelination, axonal degeneration and astrogliosis. Although the role of the immune system in the onset of MS is debated, it is widely accepted that (auto-) immune responses contribute to progression of MS. Based on comparison with the animal model experimental allergic encephalomyelitis (EAE), $CD4^+$ T cells are thought to be essential for the formation of MS lesions (Hartung and Rieckmann, 1997). In addition, other components of the immune system, such as antibodies directed against myelin proteins, were shown to contribute to the severity of EAE by enhancing CNS inflammation and demyelination (Morris-Downes *et al.*, 2002; Lyons *et al.*, 2002).

One of the most common immunological abnormalities in MS patients is enhanced intrathecal production of antibodies, suggesting that antibodies may also play a role in MS (Correale and de los Milagros Bassani Molinas, 2002). An immunohistochemical study of lesion material of acute MS patients showed deposition of antibodies in fifty percent of MS patients, and it was suggested that the presence of antibodies in these lesions represents an immunopathological subtype of MS where antibodies are relevant (Lucchinetti *et al.*, 2000). Isolation of antibodies from MS tissue revealed that at least part of the antibodies in MS lesions is directed against the major myelin protein, myelin basic protein (MBP) (Warren and Catz, 1993). In addition, EM studies demonstrated that antibodies directed against MBP and myelin oligodendrocyte glycoprotein (MOG) were present at the demyelinating edge of active MS lesion. In fact, anti-MBP and anti-MOG antibodies colocalised with disintegrating myelin sheaths, but also with ingested myelin within phagocytic cells, suggesting a role for anti-myelin antibodies in myelin phagocytosis (Genain *et al.*, 1999).

If anti-myelin antibodies do contribute to demyelination in (a subtype of) MS, pathogenic antibodies could be a target for immune therapy, such as intravenous immunoglobulins (IVIg), which has shown beneficial effects in patients with relapsing remitting MS (Fazekas *et al.*, 1997). An essential requirement for such therapy is that patients with putative antibody mediated inflammation can be identified with relatively simple methods.

In search of a biomarker for antibody-mediated pathology in MS, serum of MS patients has been studied extensively for the presence of antibodies directed against a range of myelin antigens, including MBP, MOG, proteolipid protein (PLP) and oligodendrocyte specific protein (OSP) (Sun *et al.*, 1991; Bronstein *et al.*, 1999; Reindl *et al.*, 1999; Vojdani *et al.*, 2003). Enhanced anti-myelin antibody responses have been described in subgroups of MS patients; however anti-myelin antibodies were often not specific for MS and no single antigen has been associated exclusively with MS. Analysis of antibody responses to three different myelin proteins in a single study demonstrated that elevated antibody levels to all three proteins were observed in MS patients, but that antibody responses to individual myelin proteins were not necessarily correlated (Vojdani, 2003). This suggests that it may be valuable to test antibody responses to multiple myelin antigens at the same time.

We recently described an assay that detects antibodies directed against human whole myelin, including MBP, PLP and MOG (Chapter 6 of this thesis). The assay detects antibodies directed against native, conformational myelin antigens. This is highly relevant, as *in vivo* studies demonstrated that antibodies directed against such epitopes can mediate demyelination and contribute to disease progression, whereas antibodies directed against linear epitopes were

not pathogenic (von Budingen *et al.*, 2004). The myelin flow cytometry assay identified enhanced anti-myelin IgG responses in almost fifty percent of MS patients.
In the present study, we measured anti-myelin antibody responses in another, larger group of MS patients and healthy donors. Furthermore, the clinical relevance of anti-myelin antibodies was addressed by correlating anti-myelin antibody levels to clinical disability measured by the expanded disability status scale (EDSS) and multiple sclerosis functional composite scale (MSFC). In addition, we compared the anti-myelin antibody response to the anti-MOG antibody response in the same population of MS patients and healthy donors.

Materials and Methods

Patient characteristics

Serum was obtained from 99 MS patients and 40 healthy donors (HD). Fifty four patients had relapsing remitting MS (RRMS), 21 patients had secondary progressive MS (SPMS) and 18 patients had primary progressive MS (PPMS). Of 6 MS patients the disease subtype was unknown. Patient characteristics are summarized in table 1. Disability was measured by trained neurologists using the expanded disability status scale (EDSS) (Kurtzke, 1983) and the Multiple Sclerosis Functional Composite (MSFC) (Rudick *et al.*, 2002). EDSS and MFSC scores of MS patients are indicated in table 1. The study was performed with approval of the local ethical committee and in accordance with the Helsinki Declaration.

Table 1. Patient characteristics

Subjects (n)	Age (range)[a]	Females (%)	Disease duration (range)[b]	EDSS	MSFC
MS patients (99)	44 (18-79)	61	9.2 (0.6-25.8)	4 (0-8)	0.5 (-2.3-1.4)
RRMS (54)	36 (18-61)	65	6.4 (0.6-25.8)	2.5 (0-6.5)	0.7 (-0.7-1.4)
SPMS (21)	47 (30-72)[c]	57	11.7 (4.4-31.0)[e]	6.0 (1.5-7.5)[g]	-0.2 (-2.3-0.7)[h]
PPMS (18)	56 (33-79)[d]	56	17.5 (5.1-39.2)[f]	6.0 (2.5-8.0)[g]	0.2 (-1.6-0.9)[h]
Healthy donors (40)	39 (29-62)	53			

[a]Median age in years
[b]Median disease duration in years
[c]SPMS higher than RRMS (p<0.001) and healthy donors (p<0.01)
[d]PPMS higher than SPMS (p<0.05), RRMS (p<0.001) and healthy donors (p<0.001)
[e]SPMS longer than RRMS (p<0.05)
[f]PPMS longer than RRMS (p<0.001)
[g]SPMS and PPMS higher than RRMS (p<0.001)
[h]PPMS and SPMS lower than RRMS (p<0.001)

Isolation of human whole myelin

Human CNS white matter tissue was obtained at autopsy from individuals without a history of neurological disease, in collaboration with the Dutch Brain Bank (coordinator: Rivka Ravid). Myelin was isolated according to the method of Norton and Poduslo (Norton and Poduslo, 1973), with minor adaptations. Briefly, CNS white matter was homogenized in 0.32 M sucrose. The suspension was layered over a 0.85 M solution of sucrose and centrifuged at 75,000 g for 30 min at 4°C (step 1). The interphase, containing the myelin, was pooled and washed three times with de-ionized water by repeated centrifugation at 75,000 g for 15 min at 4°C (step 2). Steps 1 and 2 were repeated twice. Myelin was collected and stored at –20°C for use.
The total protein concentration of isolated myelin was calculated using a bovine serum albumin (BSA) standard curve as described (Van der Goes *et al.*, 1999). Myelin from five subjects was pooled for use in all assays.

Myelin Flow Cytometry Assay

Myelin, isolated as described above, is a suspension of small vesicles in aqueous solution, allowing detection of (membrane) myelin antigens by flow cytometry (Chapter 6).

Human myelin (15 μg) and undiluted serum (4 μl) were added to 100 μl PBS in a 96-well V-bottom plate and incubated for 30 min at 37°C or overnight at 4°C. Unbound serum proteins were removed by washing the myelin in PBS (4,500 rpm, 4 min, 3 times repeated). Myelin was subsequently incubated with polyclonal rabbit anti-human Ig (detecting a combination of human IgG, IgM and IgA; DAKO, Glostrup, Denmark) for 30 min at 37°C. After washing in PBS, samples were incubated with Phycoerythrin (PE)-labelled donkey anti-rabbit Ig (Jackson Laboratories, West Grove, PA, US), washed again and taken up in 50 μl PBS in FACS tubes (B&D Biosciences, Franklin Lakes NJ, US). Alternatively, biotinylated goat-anti-human IgG or biotinylated donkey-anti-human IgM (Jackson Laboratories) were used as a secondary antibody to specifically detect binding of anti-myelin IgM or IgG. After washing, samples were incubated with Alexa$^{488/594}$-conjugated streptavidin (Molecular Probes, Eugene, OR, US), washed and taken up in 50μl PBS in FACS tubes.

As a positive control, human myelin was incubated with 2μg/μl monoclonal antibodies directed against MOG (Z12 mAb, kindly provided by Dr. Piddlesden (Piddlesden *et al.*, 1993). Binding of Z12 mAb was detected using PE-labelled rabbit-anti-mouse Ig (Jackson Laboratories). As a negative control myelin was directly incubated with conjugate antibody only.

Myelin immunoreactivity was measured using the FACS Calibur (B&D Biosciences, Franklin Lakes NJ, US). Anti-myelin antibody responses were expressed using the Mean Fluorescence Intensity (MFI). All serum samples were incubated with myelin in triplicate and all samples were included in at least three separate experiments.

Recombinant MOG ELISA

Recombinant protein corresponding to the N-terminal sequence of rat MOG (rrMOG, amino acids 1-125), was expressed in *E. coli* and purified by metal chelate chromatography as described (Amor, 1994). Purified protein was dialyzed into PBS and stored at -20ÚC. Recombinant protein corresponding to the human MOG sequence rhMOG, amino acids 1-125), was a kind gift from Dr. Weissert (University of Tübingen, Tübingen, Germany).

Ninety-six well ELISA plates (Nunc, Roskilde, Denmark) were coated with 5 μg/ml rrMOG or rhMOG diluted in phosphate buffered saline (PBS, pH 7.4) (100μl/well). After overnight incubation at 4°C, plates were washed in PBS/0.1% Tween and blocked with PBS/1%BSA (37°C, 1 h). After a washing, diluted serum samples (1:200) were added to the plates in duplo and incubated for 1 h at 37°C. Plates were washed again to remove unbound antibodies, and incubated peroxidase (PO)-labelled rabbit anti-human Ig to detect binding of IgG, IgM and IgA antibodies (DAKO, Glostrup, Denmark), or with biotinylated goat-anti-human IgG or biotinylated donkey-anti-human IgM (Jackson Laboratories) followed by incubation with PO-labelled streptavidin (Vector Laboratories, Burlingame, CA, US) to specifically detect IgG or IgM antibodies. After a final washing step, binding of secondary antibodies was or streptavidin was detected by adding substrate (OPD, Merck-Schuhardt, München, Germany). After 10-20

minutes the reaction was stopped using 1 M H_2SO_4 and optical density of the wells was measured in an ELISA plate reader at 490 nm.

Statistical analysis

To compare anti-myelin antibody responses in MS patients and donors, results obtained in representative experiments were pooled as described in Chapter 6.

Differences in median age and disease duration between groups (MS patients, MS clinical subgroups and HD) were analysed using Mann-Whitney U-test. Differences in the distribution of females and males in MS patients and HD were analysed using Chi-square test. Anti-myelin antibody responses in MS patients and HD were analysed using the Mann-Whitney U-test.

Anti-myelin antibody levels in individual MS serum samples were considered to be enhanced over HD values when the MFI was higher than the [average value of HD + 2.5*standard deviation]. Possible differences in the proportion of anti-myelin Ig positive samples between clinical subgroups of MS patients were analysed using the Chi-square test. P values lower than 0.05 were considered to indicate statistical significance (confidence level 95%).

Results

MS patients show enhanced levels of anti-myelin IgG and IgM

The average anti-myelin Ig level (total Ig) in MS patients (n=98) was not enhanced when compared to healthy donors (HD, n=40), although a number of MS patients with a relatively high anti-myelin antibody response were identified among the MS patients but not among HD (Figure 1). No significant differences in the anti-myelin Ig response were observed between clinical subgroups of MS patients (data not shown).

When analysed separately, both anti-myelin IgG and anti-myelin IgM levels were significantly higher in MS patients than in HD (both IgG and IgM: MS vs. HD, $p<0.001$) (Figure 1). Enhanced anti-myelin IgG and IgM responses were observed in RRMS, SPMS and PPMS patients with no significant differences between clinical subgroups (all clinical subgroups vs. HD, $p<0.001$ for IgG and $p<0.05$ for IgM).

Individual analysis of MS serum samples revealed that 6/98 MS patients showed an anti-myelin Ig response that was enhanced over HD (cut-off value: average HD + 2.5*standard deviation), whereas all HD showed values lower than the cut-off value (table 2). The proportion of MS patients that showed enhanced anti-myelin IgG was 44/99; the anti-myelin IgG response

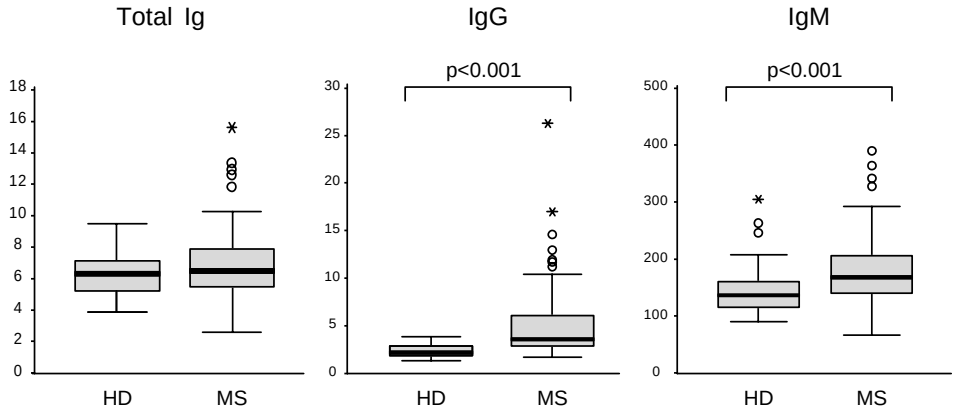

Figure 1.

Enhanced IgG and IgM antibodies directed against human whole myelin are detected in MS patients. Anti-myelin total Ig antibody levels were comparable in MS patients and healthy donors (HD) (left panel). MS patients show enhanced anti-myelin IgG, whereas the anti-myelin IgG response in HD is homogeneously low (middle panel). The anti-myelin IgM response is heterogeneous in HD and MS patients, although anti-myelin IgM levels in MS are significantly enhanced over HD (right panel). Boxes extend from the 25th to the 75th percentiles, the middle line represents the median and bars show the range of the data. Outliers (o) showed MFI values extending between 1.5 and 3 box lengths from the upper edge of the box. Extremes (*) represent cases with MFI values more than 3 box lengths form the upper edge of the box.

of all HD was lower than the cut-off value. Anti-myelin IgM was enhanced in 8/99 MS patients but also in 2/38 HD, showing that for anti-myelin IgM responses, outliers are identified among HD as well as MS patients. Again, no significant differences were observed between clinical subgroups of MS patients (table 2).
Anti-myelin Ig, IgG or IgM responses were not correlated with the age of either HD or MS patients (data not shown).
Data are representative of at least three different experiments.

Table 2: MS patients and healthy donors showing enhanced anti-myelin and anti-MOG antibody responses

	Whole myelin			Recombinant MOG		
	Total Ig	IgG	IgM	Total Ig	IgG	IgM
MS patients	6/98	44/99	8/99	2/99	3/99	9/99
RRMS	4/53	22/54	5/54	1/54	2/54	4/54
SPMS	1/20	11/20	2/14	0/21	0/21	3/21
PPMS	0/17	5/17	0/17	0/18	1/18	0/18
Healthy donors	0/40	0/38	2/38	0/40	1/40	1/40

Anti-myelin IgG and IgM are not related to disease duration or disease severity

Anti-myelin total Ig correlated weakly with disease duration (Spearman's Rho=0.25, $p<0.05$). However, no correlation between disease duration and anti-myelin IgG or IgM levels was observed (data not shown). Anti-myelin Ig, IgG or IgM responses were not related to clinical severity of disease, as measured by EDSS or MSFC (data not shown).

MS patients and HD show similar antibody responses to recombinant MOG

Antibody responses to recombinant MOG were measured in MS patients and HD. At the group level, anti-myelin Ig, IgG and IgM responses were not different between MS patients and HD (Figure 2). The OD values for anti-MOG IgG were considerably higher than for anti-MOG total Ig or IgM. It is unknown if this represents higher sensitivity of the detecting antibody or a quantitatively different IgG response.
Analysis of MS patients individually demonstrated that only 2/99 MS patients showed anti-MOG Ig values that were higher than the cut-off value [average HD + 2.5*standard deviation]. All HD showed anti-MOG Ig lower than the cut-off value. Anti-MOG IgG was enhanced in 3/99 MS patients and 1/40 HD. Anti-MOG IgM was enhanced in 9/99 MS patients and 1/40 HD. No significant differences in anti-MOG Ig, IgG or IgM were observed between the different clinical subgroups of MS patients (table 2).

The anti-MOG Ig response was weakly correlated with the age of the subjects (Rho = 0.2, $p<0.05$) and disease duration (Rho=0.3, $p<0.05$). Anti-MOG IgG levels correlated weakly with duration of disease ($p<0.05$; IgG, Rho=0.3, $p<0.05$), but not with age of the subjects. The anti-MOG IgM response was not correlated to either age or disease duration.
There were no correlations between anti-MOG antibodies (Ig, IgG and IgM) and clinical severity of MS (EDSS and MSFC) (data not shown).

Anti-myelin Ig and IgM but not IgG antibodies correlate with anti-MOG antibodies
To check if the anti-myelin antibody response was related to the anti-MOG antibody response, correlations between anti-myelin and anti-MOG antibody levels were calculated. The anti-myelin Ig response correlated weakly but significantly with the anti-MOG Ig response (Spearman's Rho = 0.2, $p<0.01$). The correlation between anti-myelin IgM and anti-MOG IgM was stronger and more significant (Spearman's Rho = 0.5, $p<0.0001$). In contrast, the anti-myelin IgG response was not correlated to the anti-MOG IgG response.

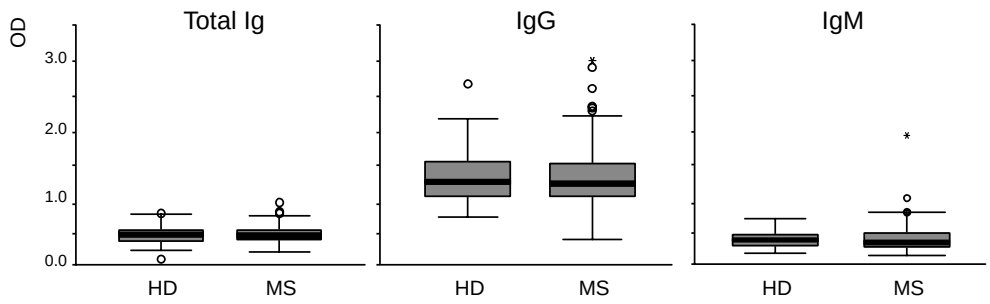

Figure 2.
Comparable levels of anti-MOG total Ig, IgG and IgM in MS patients and healthy donors (HD). Boxes extend from the 25th to the 75th percentiles, the middle line represents the median and bars show the range of the data. Outliers (o) showed MFI values extending between 1.5 and 3 box lengths from the upper edge of the box. Extremes (*) represent cases with MFI values more than 3 box lengths form the upper edge of the box. A higher number of outliers and extremes are observed among MS patients for anti-MOG total Ig, IgG and IgM antibodies. Differences between MS patients and HD were not significant.

Discussion

We previously described an assay to detect serum antibodies directed against whole human myelin (Chapter 6 of this thesis). In the present study, we expanded the analysis of anti-myelin antibody responses in MS by analysing a larger group of MS patients and healthy donors, by correlating anti-myelin antibody responses to clinical parameters such as disease severity and disease duration and by comparing anti-myelin antibody responses to anti-MOG antibody responses.

Anti-myelin total Ig levels in MS patient serum were not significantly different from anti-myelin total Ig in HD serum. In contrast, the anti-myelin IgG was clearly enhanced in MS patient serum whereas the anti-myelin IgG response in HD was homogeneously low. The anti-myelin IgM response was heterogeneous in both MS patients and HD, although overall anti-myelin IgM levels in MS were higher than in HD.

These results support our previous results, obtained in another, smaller population of MS patients, that enhanced levels of anti-myelin IgG are observed in (a subgroup of) MS patients. The proportion of MS patients showing anti-myelin IgG levels higher than HD was forty five percent in this study and forty seven percent in the previous study, demonstrating that this is a robust observation. In contrast to the previous study, we here observe enhanced anti-myelin IgM in MS serum. However, enhanced anti-myelin IgM responses were observed in individual MS patients as well as HD. This emphasizes that elevated anti-myelin IgM responses are not specific for MS.

It is unclear why anti-myelin total Ig levels in MS patients did not exceed those of HD, while anti-myelin IgG and IgM levels did. Enhanced anti-myelin IgM and IgG responses may be obscured by anti-myelin IgA responses that do not differ between MS patients and HD. However, this is unlikely as elevated serum IgA levels specific for a number of myelin proteins have been described in MS (Vojdani *et al.*, 2003; Kennel De March *et al.*, 2003). Anti-myelin IgA responses have not been studied thus far. Another possibility is that the myelin flow cytometry assay is less sensitive when a mixed conjugate (specific for IgG, IgM and IgA) is used instead of a conjugate that is specific for one antibody isotype, as a result of higher background staining when a less specific conjugate is used.

A characteristic of the antibody response in MS is the persistence of the response; this is in contrast to the usually transient production of (anti-myelin) antibodies in other neurological diseases (Reindl *et al.*, 1999; Bergamaschi *et al.*, 2004). The stability of the anti-myelin antibody response over time is unknown, although pilot experiments in our laboratory suggest that follow-up samples from individual MS patients show similar responses over a period of at least nine months, in both patients with a positive and a negative anti-myelin antibody response (data not shown). In addition, anti-myelin IgG and IgM levels were not correlated with age or disease duration, suggesting that the presence anti-myelin antibodies does not reflect accumulation of antibody responses directed against myelin proteins over time, as has been suggested for the anti-MBP IgG response (Reindl *et al.*, 1999).

Although the anti-myelin IgG response was related to a subpopulation of MS patients, this could not be attributed to any of the clinical subtypes of MS. Nevertheless, it is possible that

the subpopulation of MS patients showing enhanced anti-myelin IgG reflects a immunopathological subtype of MS, as it has been proposed that clinical subtypes of MS patients based on disease course do not reflect the heterogeneity in underlying immunopathological processes (Lucchinetti *et al.*, 2000).

A recent study suggests that the presence of serum anti-MOG antibodies in patients with clinically isolated syndrome predicts rapid progression to clinically definite MS (Berger *et al.*, 2003). These are very promising results, suggesting that testing anti-myelin antibodies may provide an easy tool to select MS patients for early treatment. However, the pathogenic relevance of the antibodies measured in the study by Berger *et al.* is unclear, as the 'predictive' antibodies were detected using Western blot, detecting antibodies against linear epitopes of recombinant MOG (Berger *et al.*, 2003). Antibodies against linear MOG epitopes are qualitatively different from antibodies against conformational epitopes (Haase *et al.*, 2001). Whereas antibodies against conformational epitopes could induce lysis of MOG-transfected cells and exacerbate EAE, antibodies against linear epitopes were not pathogenic (Bourquin *et al.*, 2003; von Budingen *et al.*, 2004). The anti-myelin antibodies measured in the present study were directed against native, conformational myelin antigens and may thus represent pathogenically relevant antibodies.

It is unknown if MS patients with evidence of antibody-mediated pathology in active demyelinating lesions, as described by Lucchinetti *et al.* (2000), show enhanced serum anti-myelin antibody serum. This should be subject of further study. In addition, future studies are required to address the predictive value of anti-myelin antibodies for MS disease progression and the *in vitro* pathogenic capacity of anti-myelin antibodies isolated form MS serum samples. The enhanced anti-myelin IgG and IgM levels in MS patients were not reflected in anti-MOG antibody levels, as the anti-MOG total Ig, IgG and IgM response were comparable in MS patients and healthy donors. This supports a number of earlier studies (Xiao *et al.*, 1991; Lampasona *et al.*, 2004), although enhanced anti-MOG IgG and IgM levels in MS patient serum have often been described as well (Lindert *et al.*, 1999; Vojdani *et al.*, 2003). The inconsistent results in studies on anti-MOG antibodies probably reflect different methods and different MOG preparations used in different studies, as discussed before (Chapter 6).

Although anti-MOG antibody responses were not significantly enhanced in MS patients, there was a significant correlation between the levels of anti-MOG total Ig and IgM and the levels of anti-myelin total Ig and IgM, suggesting that the anti-myelin antibody response may partly represent antibody responses to MOG. However, the possibility that both anti-myelin and anti-MOG Ig and IgM responses reflect total immunoglobulin concentration in serum cannot be excluded, as the total antibody levels of the sera were not measured. Importantly, the anti-myelin IgG response did not correlate with the anti-MOG IgG response. This, in addition to the fact that anti-myelin IgG was more specific for MS than anti-myelin IgM, suggests that the anti-myelin IgG response may be directed against qualitatively different myelin antigens than anti-myelin IgM. This suggests the anti-myelin IgG response is dominated by antibodies directed against other myelin antigens or to MOG epitopes that are not represented in recombinant MOG. It must be noted that anti-MOG antibodies were detected using an ELISA, recognising antibodies directed against both linear and conformational, but

not post-translationally modified, epitopes of recombinant MOG. It is possible that anti-myelin IgG responses in MS patients may be partly directed against glycosylated MOG epitopes, as was recently suggested (Gaertner *et al.*, 2004).

In summary, the present study demonstrates that enhanced levels of IgG antibodies directed against human whole myelin are present in approximately fortyfive percent of MS patients, confirming an earlier study in a another, smaller population of MS patients. The anti-myelin IgG response is not correlated with the clinical subtype of MS, clinical severity of MS, disease duration or the age of the subjects. This suggests that the presence of anti-myelin antibodies may define a subgroup of MS patients where antibody-mediated mechanisms contribute to pathogenesis or disease progression. Extensive efforts to identify antibody responses to individual myelin antigens have thus far not yielded antigen specificity specifically associated with MS, or provided a biomarker to identify MS patients with antibody-mediated disease. Therefore the most relevant question is not the protein specificity of anti-myelin antibodies, but more importantly the pathogenic relevance of anti-myelin antibodies. This could be done by addressing the capacity of anti-myelin antibodies, isolated from MS patient serum, to fix complement or cross-link macrophages Fc³ receptors, two parameters that were shown to be highly relevant for pathogenic capacity of anti-myelin antibodies EAE (Piddlesden *et al.*, 1993; Van der Goes *et al.*, 1999; <[04] Authors, primary>, <[05] Date, primary>). Eventually, such studies could help to elucidate the relevance of anti-myelin antibody responses in MS, or the development of a biomarker to identify MS patients that may benefit from immunotherapy with autoantibodies as a target.

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Chapter 8

Summary and discussion

Summary and discussion

The role of humoral immunity in Multiple Sclerosis has been a subject of debate for many years. Recent developments in the field suggest that antibodies and complement are involved in MS lesion formation in a subgroup of patients, and that anti-myelin antibodies have predictive value for the rapid development of relapses after a first clinical episode of MS (Lucchinetti *et al.*, 2000; Berger *et al.*, 2003). However, it is unclear if anti-myelin antibodies actually contribute to MS pathogenesis or merely represent an epiphenomenon that develops in response to continuous myelin breakdown. In fact, B cells have even been hypothesized to play a regulatory role in the chronic phase of experimental demyelinating disease (Fillatreau *et al.*, 2002).

If anti-myelin antibodies actively contribute to CNS inflammation and demyelination, they could form a target for therapy, especially if relevant antibody responses can be detected early in disease when immunotherapy is most successful (Rizvi and Agius, 2004). In addition, anti-myelin antibodies may represent a valuable biomarker to predict disease progression as has been suggested recently (Berger *et al.*, 2003), even if they are a harmless by-product of demyelinating disease.

It is not easy to unravel the immunological processes that contribute to MS. As the aetiology of the disease is unknown, it is difficult to pinpoint disease onset because the first clinical presentation of MS may be preceded by a long period of subclinical pathological events. In addition, at least part of the immune response is thought to take place locally in the CNS, a compartment that is not easy accessible for the isolation of body fluids or biopsy material, limiting the possibilities for large-scale clinical studies. Finally, due to the chronic nature of the disease, autopsy material often represents later stages of disease where the factors contributing to the onset of disease may be obscured by secondary processes. For these reasons, most hypotheses on the immunological events underlying MS are based on experimental models *in vitro* and *in vi*vo. Using these models it was shown that anti-myelin antibodies enhance myelin phagocytosis and the release of inflammatory mediators *in vitro* by activating the complement system and crosslinking of macrophage FcγR receptors (**Chapter 1**). *In vivo* studies in different models of experimental allergic encephalomyelitis (EAE) demonstrated that anti-myelin antibodies were unable to induce EAE in absence of an encephalitogenic T cell response, but that anti-myelin antibodies could contribute to the effector phase of EAE by enhancing CNS inflammation and demyelination (Linington *et al.*, 1988). Later studies in B cell deficient mice showed that B cells were not essential for the induction of EAE, although anti-myelin antibodies could provide the crucial additive effect if the encephalitogenic T cell response was weak (Lyons *et al.*, 2002). Similarly, complement activation was not essential for the induction of EAE although milder induction protocols were less efficient at inducing clinical EAE in complement deficient animals (Nataf *et al.*, 2000; Calida *et al.*, 2001) (**Chapter 2**).

The capacity to exacerbate clinical signs of EAE was mostly restricted to antibodies directed against MOG and the antibodies that could fix complement most efficiently had the highest demyelinating capacity (Piddlesden *et al.*, 1993). However, complement depletion could never

completely block anti-MOG antibody mediated exacerbation of EAE (Piddlesden *et al.*, 1991; Morris-Downes *et al.*, 2002). Moreover, antibodies with potent *in vivo* demyelinating capacity could enhance myelin phagocytosis *in vitro* in absence of active complement, presumably through FcγR mediated phagocytosis (Van der Goes *et al.*, 1999). Therefore we hypothesized that anti-MOG antibodies contribute to disease exacerbation at least partly through interactions with the activating FcγR, FcγRI and FcγRIII. **Chapter 3** demonstrates that the rapid exacerbation of EAE by anti-MOG antibodies is independent of FcγR-IgG interactions as injection of antibodies rapidly enhanced mortality in both wild type and FcRγ$^{-/-}$ chain deficient mice. Therefore it is likely that the acute effect of anti-MOG antibodies is mediated by activation of the classical pathway of complement. The reported inability of complement depletion to fully block anti-MOG antibody mediated exacerbation of EAE could be related to the transient release of inflammatory mediators that is associated with CVF treatment. This is supported by the protective effect of soluble complement receptor and C6 deficiency in antibody-exacerbated EAE in rats (Piddlesden *et al.*, 1994; Mead *et al.*, 2002). Further studies of antibody exacerbated EAE in complement deficient mice, particularly C1q knockout mice, should be able to unravel the role of complement activation pathways in antibody-mediated demyelination. The inability of both complement depletion and FcγR deficiency to suppress antibody mediated exacerbation of EAE could also indicate redundancy of complement- and FcγR-mediated pathways as has been shown in other models of antibody-mediated inflammation (Trcka *et al.*, 2002).

The other important observation in **Chapter 3** is that FcRγ$^{-/-}$ mice are fully susceptible to MOG35-55 induced EAE. This in accordance with previous reports showing that the induction of EAE was independent of B cells (Lyons *et al.*, 1999), but in contrast to more recent observations that FcRγ$^{-/-}$ mice are resistant to induction of EAE by both recombinant rat (rrMOG) and peptide MOG (MOG35-55) (Lock *et al.*, 2002; Abdul-Majid *et al.*, 2002). Given the fact that EAE can be induced by transfer of encephalitogenic T cells and by active immunisation in several strains of B cell deficient mice, it is highly unlikely that IgG-FcγR interactions should be essential for the induction of EAE. The absence of EAE in FcRγ$^{-/-}$ mice in other studies, and the delayed onset in our study is probably unrelated to absent surface expression of FcγRI and FcγRIII, but the result of aberrant FcRγ chain signalling in other receptor complexes (Arase *et al.*, 1997; Takai and Ono, 2001; Wu *et al.*, 2001). This is supported by unpublished studies performed in our laboratory on MOG35-55 EAE in FcγRIII$^{-/-}$ mice that lack FcγRIII expression due to deletion of the FcγRIII alpha chain but have unimpaired FcRγ chain function (Hazenbos *et al.*, 1996). FcγRIII$^{-/-}$ mice developed EAE with similar clinical and pathological characteristics as wild type mice, including normal onset of EAE (unpublished results). Interestingly, another group reported attenuated EAE in FcγRIII$^{-/-}$ mice (Pedotti *et al.*, 2003), suggesting that functional expression of FcγRIII, and not just the FcRγ chain, may contribute to the induction of EAE. The FcγRIII$^{-/-}$ chain mice used in this study and in our study were originally from the same strain, although the mice used in our study were backcrossed to the C57BL/6 background for more generations (twelve generations in our study, versus six generations in the study by Pedotti *et al.*). It is unclear why the results in the two laboratories are different, even if the EAE immunisation protocols were

highly similar. The differences between the results obtained in FcR$\gamma^{-/-}$ chain mice in the different laboratories are even more striking (**Chapter 3**).

These discrepancies reflect the sensitivity of the EAE model for changes in the experimental setting, including genetic background, gender and age of the mice (Voskuhl *et al.*, 1996b; Abdul-Majid *et al.*, 2000), but also less obvious changes such as the season of immunisation (Teuscher *et al.*, 2004) and most likely the infectious state of the animal facility. In addition, mice backcrossed from the 129 background - a popular strain for the generation of knockout mice - to other genetic backgrounds may have unpredictable phenotypes (Bygrave *et al.*, 2004). We have experienced this in our own hands, when FcγRI/III double knockout (FcγRI/III$^{-/-}$) mice of mixed background (129/C57BL6/BalbC, H-2^{b}), with an intact FcRγ chain, showed attenuated MOG35-55 EAE whereas in the same experiment, FcR$\gamma^{-/-}$ and FcRγIII$^{-/-}$ C57BL/6 (H-2^{b}) mice were fully susceptible (Box 8.1.). Although these results were very intriguing, we decided not to publish the results obtained in the FcγRI/III$^{-/-}$ mice, since a proper control strain with the same genetic background was lacking and it was unclear to what genetic background the EAE phenotype of the FcγRI/III$^{-/-}$ could be attributed. Instead we took advantage of the observation that FcR$\gamma^{-/-}$ C57BL/6 mice, the genetic background of which was well defined as they were generated on the C57BL/6 background (Takai *et al.*, 1994), showed full susceptibility to EAE to study the role of IgG-FcγR interactions in antibody mediated demyelination.

The variability of the EAE model between laboratories and experiments may nicely reflect the biological variance in (auto-) immunity even in inbred animals; however, in a laboratory setting it is important that conclusions should be based on reproducible induction protocols and well defined animal strains. It is therefore of great importance that the models that are chosen to study (particular aspects of) CNS inflammation and demyelination are selected very cautiously.

The direct effect of anti-MOG antibody injection of in MOG35-55 EAE was independent of FcγR-IgG interactions. However, immunohistochemical analysis of CNS tissue up to 25 days after antibody injection (35 days after immunisation) suggested that anti-MOG antibodies had a sustained effect in wild type mice but not FcR$\gamma^{-/-}$ mice (**Chapter 3**). Enhanced CNS inflammation and demyelination up to 32 days after anti-MOG antibody injection in wild type mice was also observed after injection of lower doses of Z12 mAb (125 μg instead of 1 mg), showing that this is a robust finding (unpublished pilot experiments). The absence of a long-term effect of anti-MOG antibodies in FcR$\gamma^{-/-}$ mice was based on a limited number of mice, but nevertheless it is interesting to hypothesize that FcγR-IgG interactions may contribute to the maintenance and propagation of the autoimmune response in EAE. Targeting antigens to FcγRI and FcγRIII by IC formation can greatly enhance antigen presentation, not only by more efficient antigen uptake but also by inducing dendritic cell maturation (Manca *et al.*, 1991; Rafiq *et al.*, 2002; Akiyama *et al.*, 2003). In the case of anti-MOG antibodies in EAE, this may result in enhanced T and B cell responses to MOG. Interestingly, epitope spreading, which is thought contribute to chronicity of autoimmune disease, was observed after active but not passive immunisation of mice with myelin antigens (Voskuhl *et al.*, 1996a; Tuohy and Kinkel, 2000). This suggests that factors other than activated T cells, possibly myelin

Box 8.1. EAE is attenuated in mice lacking activating FcγRI and FcγRIII

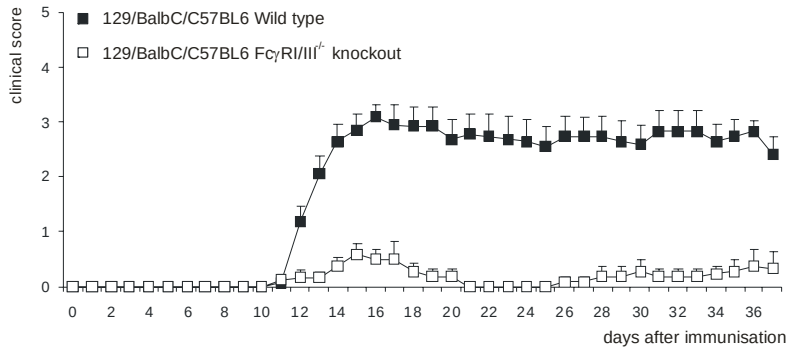

Figure 1.
Clinical course of EAE in 129/BalBC/C57BL6 Wt and FcγRI/III$^{-/-}$ mice.
Animals were immunised with MOG35-55 according to the protocol described in Chapter 3. Filled squares represent Wt mice (n=19), open squares represent FcγRI/III$^{-/-}$ mice (n=20). Error bars indicate s.e.m.

Table 1. MOG35-55 EAE in 129/BalbC/C57BL6 Wt and FcγRI/III$^{-/-}$ mice

Mice (n)	Incidence	Day of onset (±stdev)	Maximal score (±stdev)[b]
129/BalbC/C57BL6 Wt	19/19	12.7 (1.6)	3.3 (1.1)
129/BalbC/C57BL/6 FcRγI/III$^{-/-}$	8/20[a]	16.3 (7.3)	1.6 (0.9)[c]

[a] Significantly lower than Wt mice ($p<0.001$, Pearson Chi-square)
[b] Mice with no clinical signs of EAE were excluded from the calculation
[c] Significantly lower than Wt mice ($p<0.001$, Student's t-test)

specific B cell responses, may contribute to determinant spreading. The capacity of IgG-FcγR interactions to amplify the immune response in EAE may be addressed in a model of anti-MOG antibody-exacerbated EAE, where the initial immunisation is done with an antigen other than MOG, such as chronic relapsing EAE induced by PLP in SJL mice (Lublin *et al.*, 1981).

Interactions between anti-myelin IgG and FcγR may also contribute to MS, and thus genetic polymorphisms affecting the capacity of FcγR to interact with IgG may not only affect MS susceptibility but also disease progression. Associations between genetic polymorphisms in FcγRIIa, FcγRIIIa and FcγRIIIb have been described in diseases with a well-described antibody mediated component, such as systemic lupus erythematosis (SLE) and rheumatoid arthritis (RA) (van der Pol and van de Winkel, 1998). In **Chapter 4** we addressed the relevance of genetic polymorphisms in FcγRIIa, FcγRIIIa and FcγRIIIb for MS susceptibility and disease progression. In a large population of MS patients and healthy donors, no evidence was found that MS patients show a skewed distribution of polymorphisms in genes encoding for FcγRIIa, FcγRIIIa or FcγRIIIb in MS patients. Furthermore, FcγR polymorphisms were not associated with clinical subtypes of MS or with progression of disease over at least twenty-five years. This suggests that FcγR polymorphisms do not affect MS susceptibility or MS disease course. Another possibility is that FcγR receptors are only relevant in the subpopulation of MS patients that shows enhanced anti-myelin antibody responses, under the assumption that these antibodies are pathologically relevant. **Chapter 7** demonstrates that enhanced anti-myelin antibody levels are not restricted to one of the clinical subgroups of MS patients (RRMS, SPMS and PPMS). Thus, the subdivision of patients based on disease phase or disease course may not be the most relevant one to study FcγR polymorphisms. It would be interesting to evaluate the distribution of genetic FcγR polymorphisms in patients that showed enhanced anti-myelin IgG responses. Unfortunately, we did not yet have the opportunity to combine the analysis of anti-myelin IgG levels and FcγR polymorphisms within one population of MS patients.

In **Chapter 5** we investigated the presence of IgG, FcγR and complement in active demyelinating MS lesions in autopsy material of a large number of chronic MS patients. As active demyelinating lesions, defined by the presence of high numbers of HLA-DR$^+$ macrophages containing intracellular PLP, are rare in the CNS of chronic MS patients, earlier studies were limited to a low number of cases (Bruck *et al.*, 2001; Prineas *et al.*, 2001). Complement, IgG were consistently observed in vesicle-like structures within phagocytic cells in areas of active demyelination. Moreover, double labelling studies demonstrated that complement and IgG colocalised with PLP in phagocytic cells that also showed enhanced expression of FcγRI, FcγRII and FcγRIII. In addition, C1q and IgG colocalised in phagocytic macrophages, suggesting formation of IC. This demonstrates that all tools for antibody-mediated phagocytosis of myelin are present in active demyelinating lesions of chronic MS patients. Importantly, no heterogeneity between patients was observed with respect to deposition of antibodies and complement. This suggests that the heterogeneity that is observed in white matter lesions of early MS patients (Lucchinetti *et al.*, 2000) is not present in patients with chronic MS, at least not with respect to antibodies and complement. The selection

criteria for MS patients included in our study were chronic disease and the presence of active demyelinating white matter lesions. This resulted in a diverse population of MS patients where all clinical subtypes, RRMS, SPMS and PPMS were represented. In addition, most patients included in the study died of causes not directly related to MS. Therefore, we believe that the population of MS patients included our study is representative of chronic MS. The afore mentioned study of active demyelinating lesions in patients with early MS was largely based on biopsy material (Lucchinetti *et al.*, 2000), and therefore it is questionable to what extent this patient population is representative for early MS patients, since biopsies are only taken if a diagnosis other than MS, such as glioma, is suspected. It is an intriguing question what active demyelinating lesions in a more typical population of early MS patients would look like.

Anti-myelin antibodies were recently suggested to have predictive value for disease progression in MS. The presence of serum anti-MOG antibodies at the time of a first clinical episode (CIS) was associated with rapid progression to clinically definite MS (Berger *et al.*, 2003). The association was even stronger when both anti-MOG and anti-MBP antibodies were present. These are very promising results, as this suggests that testing of anti-myelin antibodies in serum can identify MS patients with rapid disease progression in a very early phase of disease. This could be valuable to select patients for treatment, as treatment was shown to be most effective early phase of relapsing remitting disease (Rizvi and Agius, 2004).

Although presence of anti-MOG and anti-MBP antibodies predicted rapid disease progression in the earliest phase of MS (Berger *et al.*, 2003), it is unclear to what extent these antibodies are pathologically relevant. As discussed in **Chapter 6** and **Chapter 7**, the current methods to detect anti-myelin antibodies often do not allow recognition of conformational epitopes. However, studies on antibody-exacerbated EAE in marmosets and mice demonstrated that pathologically relevant antibodies were directed against conformational epitopes whereas antibodies to linear epitopes could not enhance CNS inflammation and demyelination (Brehm *et al.*, 1999; von Budingen *et al.*, 2004). This was supported by *in vitro* experiments, where antibodies directed against conformational MOG epitopes, in contrast to antibodies recognizing linear epitopes, could lyse MOG-transfected cells (Haase *et al.*, 2001).

Therefore, we set up a new, flow cytometry-based assay to detect antibodies directed against human whole myelin (**Chapter 6**). The assay detects antibodies directed against native conformational antigens in human myelin, including MOG, MBP and PLP. Using this assay, we observed enhanced anti-myelin IgG responses in approximately fifty percent of MS patients. Analysis of another, larger population of MS patients in **Chapter 7** confirmed this initial finding. Anti-myelin total Ig (combined analysis of IgM, IgG and IgA) responses were not significantly different between MS patients and healthy donors (HD). Anti-myelin IgM responses were heterogeneous in both MS patients and HD. Anti-myelin IgG or IgM responses were not correlated to the age of the subjects, disease duration, clinical subtype of MS or disease severity. This suggests that anti-myelin antibodies do not accumulate over time in response to continuous myelin breakdown as has been suggested for individual myelin antigens (Reindl *et al.*, 1999). Moreover, analysis of follow-up samples from MS patients in the early phase of disease suggested that the anti-myelin antibody response was stable for at least two years

(pilot study in our laboratory). This suggests that anti-myelin antibodies may represent a stable serum marker that is present in a subpopulation of MS patients. The relevance of anti-myelin antibodies as a biomarker to predict disease progression is currently under investigation. The presence of serum anti-myelin IgM in MS patients correlated with the presence of anti-MOG IgM. In contrast, there was no correlation between anti-myelin IgG and anti-MOG IgG (**Chapter 7**). This suggests that the anti-myelin IgG response is directed against different myelin antigens. As anti-MOG antibodies were measured in an ELISA using recombinant MOG as an antigen, it is unclear if the anti-myelin IgG responses are directed against different myelin proteins, or against different epitopes of MOG. Results obtained in the myelin flow cytometry assay will be compared to a number of different assays to detect anti-MOG antibodies to linear, conformational and post-translationally modified epitopes in an international collaboration guided by Dr. Berger and Dr. Reindl (University of Innsbruck, Austria). In addition, the anti-myelin antibody response in MS will be compared to the anti-myelin antibody response in other inflammatory neurological diseases.

In **Chapter 6** and **7** we measured antibody responses to human whole myelin isolated from healthy donors. It has been suggested that the initial immune response in MS patients is directed against altered self-proteins. Aberrant post-translational modification of endogenous proteins, particularly changes in glycosylation and citrullination have been associated with the development of autoimmunity (van Boekel and van Venrooij, 2003; Nijenhuis *et al.*, 2004) and altered glycosylation has been hypothesised to play a role in MS pathogenesis (t Hart *et al.*, 2000). Although abnormalities in glycosylation have been described in MS, it is largely unknown if MS patients show antibody and T cell responses against differently glycosylated myelin antigens. Furthermore, the inflammatory mediators that are released in the process of demyelination, such as reactive oxygen species or proteases, may structurally change myelin proteins, creating new immunogenic myelin epitopes (Wållberg *et al*, 2005). It is unknown to what extent antibody responses in MS are directed against modified self-antigens, as these epitopes are not detected using the current methods. In the myelin flow cytometry assay, the posttranslational structure of myelin can be modified experimentally before measuring anti-myelin antibodies in MS patient sera for anti-myelin antibodies, providing a controlled system to study the antibody responses to post-translationally modified antigens. In a pilot study, we compared the serum antibody response to myelin isolated from MS patients (MS myelin) and antibody response to myelin isolated from healthy donors (HD myelin). Interestingly, MS patients but not HD showed relatively higher levels of antibodies to MS myelin than to HD myelin. This suggests that MS patients develop antibodies to myelin antigens that are associated with disease and are not present in healthy donors.

One of the most interesting questions in the study of anti-myelin antibody responses is the pathogenic relevance of these antibodies. Using immunohistochemistry, antibodies were detected in active demyelinating lesions of fifty to sixty percent of patients with early MS (Lucchinetti *et al.*, 2000), but it is unknown if these patients also present with enhanced anti-myelin antibody levels in serum. This issue will be addressed shortly, as we participate in an international study that combines immunohistochemical analysis of biopsy material with analysis of body fluids in patients with early MS.

The pathogenic relevance of anti-myelin antibodies can also be addressed using *in vitro* studies. It was previously shown that antibodies with the highest demyelinating capacity *in vivo* could efficiently fix complement and induce FcγR mediated phagocytosis *in vitro* (Piddlesden *et al*., 1993; Van der Goes *et al*., 1999). Similarly, pathogenic antibodies in human serum could possibly be identified using functional assays. It is possible that assays using functional read-outs, rather than assays measuring the mere presence of anti-myelin antibodies may be able to distinguish pathogenic from non-pathogenic antibodies. This is especially relevant for anti-myelin antibodies detected using the myelin flow cytometry assay, as these antibodies detect human myelin antigens in their native form, i.e. antigens that are present in the human CNS.

The relevance of functional assays to study pathogenic capacity of demyelinating antibodies in human disease was recently illustrated by a study of patients with the Guillain-Barré Syndrome (GBS), an acute inflammatory demyelinating disease of the peripheral nervous system. The titres of serum IgG antibodies directed against the peripheral myelin antigen GM1 could not predict their capacity to induce leukocyte activation through FcγR cross-linking, suggesting that not the serum antibody levels but the functional capacity of these antibodies to induce leukocyte effector functions is the relevant parameter to predict pathogenicity (van Sorge *et al.*, 2003). The capacity of anti-myelin antibodies isolated from MS patients to induce FcγR crosslinking and complement activation should be addressed in future studies.

In summary, the studies described in this thesis have led to the following conclusions:

- Anti-myelin antibodies have been described in CNS lesions and body fluids of MS patients, although it is unknown if and how anti-myelin antibodies contribute to CNS inflammation and demyelination in MS (**Chapter 1**)
- Antibodies and complement are not essential for in the induction of EAE although the humoral immune system may be crucial for the development of CNS inflammation and demyelination if the encephalitogenic T cell response is weak (**Chapter 2**)
- Fcγ receptors are not essential for the induction and progression of MOG-EAE or acute anti-MOG antibody mediated exacerbation of EAE. However, IgG-FcγR interactions may contribute to sustained CNS inflammation and demyelination (**Chapter 3**)
- Genetic polymorphisms in FcγRIIa, FcγRIIIa and FcγRIIIb are not associated with MS susceptibility or MS disease course (**Chapter 4**)
- Complement, immunoglobulins and FcγR are consistently associated with active demyelination in chronic MS (**Chapter 5**)
- Enhanced levels of serum IgG antibodies directed against human whole myelin are detected in approximately fifty percent of MS patients (**Chapter 6**)
- The presence of enhanced serum antibodies in MS is not related to disease duration, disease severity or the clinical subtype of MS and may represent a stable marker of antibody mediated inflammation (**Chapter 7**)

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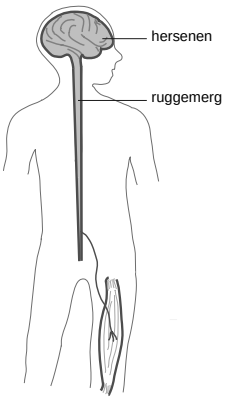
Samenvatting in het Nederlands

(voor niet-immunologen)

Index

1. Multiple sclerose

Multiple sclerose (**MS**) is een chronische ziekte die in de meeste gevallen begint bij jong volwassenen. Het belangrijkste kenmerk van MS is het ontstaan van verlammingsverschijnselen, die erger worden naarmate de ziekte vordert. Als gevolg hiervan belanden veel MS patiënten uiteindelijk in een rolstoel.
Hoewel MS effect heeft op het functioneren van de spieren is het geen spierziekte. De verlammingen zijn het gevolg van een complex proces in de hersenen en het ruggenmerg.



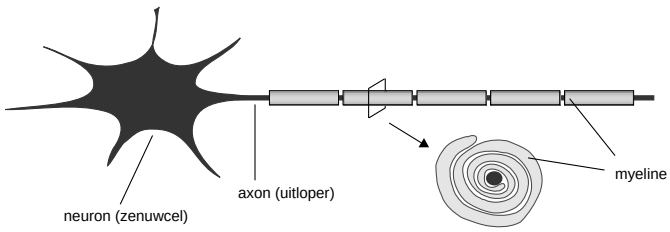
Figuur 1

1.1. MS: een ziekte van het centraal zenuwstel

De hersenen en het ruggemerg vormen samen het centraal zenuwstelsel (**CZS**) (Figuur 1). Het CZS heeft een belangrijke regeltaak in het lichaam. Het regelt bijvoorbeeld het aanspannen van de spieren in de rest van het lichaam, waardoor we in staat zijn onze ledematen te bewegen. Het CZS bestaat uit vele **neuronen** (ook wel 'zenuwcellen' genoemd) die elektrische signalen afgeven. Neuronen hebben lange uitlopers (**axonen**) die het signaal vanuit de neuronen door het ruggemerg naar andere delen van het lichaam geleiden, bijvoorbeeld naar de spieren. Als je besluit je been op te tillen, gaat er op die manier een elektrisch signaal vanuit de hersenen naar de beenspier, waardoor de spier samentrekt en het been gebogen wordt. Om het signaal op efficiënte manier door te geleiden zijn de axonen ingepakt in een isolatielaag. Vergelijk het met een elektriciteitsdraad die wordt beschermd door een kunststof omhulsel zodat hij niet zo snel beschadigd raakt, en zodat het elektrische signaal niet kan 'weglekken'. Bij neuronen bestaat de isolatielaag uit een samenstelling van vetten en eiwitten, het **myeline** (Figuur 2).

1.2. Wat gaat er mis bij MS?

In de hersenen van MS patiënten raakt myeline beschadigd (**demyelinisatie**). De overdracht van signalen in de hersenen wordt hierdoor minder efficiënt, en de kwetsbare axonen lopen gemakkelijk beschadiging op. Demyelinisatie ontstaat vaak op meerdere ('multiple') plaatsen in het CZS. Op de plaatsen (ofwel **laesies**) wordt myeline uiteindelijk vervangen door littekenweefsel ('sclerose'). Demyelinisatie en beschadiging van axonen leiden ertoe dat signalen vanuit de hersenen de spieren niet meer kunnen bereiken, waardoor MS patiënten hun spieren niet goed meer kunnen gebruiken. Ze raken dan verlamd, terwijl er aan de spieren zelf eigenlijk niets mankeert.
Het is nog niet precies bekend wat de demyelinisatie veroorzaakt. Artsen en onderzoekers vermoeden dat de oorzaak van demyelinisatie niet bij alle MS patiënten dezelfde is. Niettemin



Figuur 2.
Neuronen hebben lange uitlopers, de axonen, die zijn ingepakt in myeline (gemyeliniseerd). De onderste tekening is een dwarsdoorsnede van een gemyeliniseerd axon.

zijn er veel aanwijzingen dat een verstoring van het immuunsysteem een belangrijke factor is bij een grote groep MS patiënten.

1.3 Het immuunsysteem

Het immuunsysteem zorgt voor de afweer van het lichaam tegen ziekteverwekkers, zoals virussen en bacteriën. Het immunsysteem is in staat ziekteverwekkers te herkennen en op te ruimen (**de immuunrespons**), waardoor infectieziektes in de kiem gesmoord worden.
Witte bloedcellen en antistoffen (ofwel **antilichamen**) zijn twee belangrijke wapens in de strijd tegen ziekteverwekkers.
T cellen, **B cellen** en **macrofagen** zijn drie verschillende soorten witte bloedcellen die elk hun eigen manier hebben om ziekteverwekkers te lijf te gaan. Als er geen gevaar dreigt circuleren de witte bloedcellen door het lichaam, ze zijn dan in inactieve staat (Figuur 3A). Als een T cel een ziekteverwekker tegenkomt, raakt hij actief en begint de ziekteverwekker onschadelijk te maken. Ook B cellen raken geactiveerd als ze in aanraking komen met ziekteverwekkers. Zij zijn echter niet in staat de ziekteverwekker direct uit te schakelen, maar scheiden in plaats daarvan **antilichamen** uit. Dit zijn kleine eiwitstructuren die specifiek plakken aan ziekteverwerkers (Figuur 3A). Ze dienen als een ‘vlaggetje’ dat wordt herkend door weer een andere soort witte bloedcel, de macrofaag. Macrofagen kunnen zelf nauwelijks ziekteverwekkers herkennen, maar ze zijn erg goed in het herkennen van antilichamen (de vlaggetjes) die gebonden zijn aan ziekteverwekkers. Als een macrofaag een antilichaam herkent dat aan een ziekteverwekker geplakt is, ‘eet’ hij het complex van ziekteverwekker en antilichaam snel en efficiënt op (**fagocytose**) (macrofaag betekent letterlijk ‘grote eter’) (Figuur 3B).
Ook **complementeiwitten** zijn in staat de ‘vlaggetjes’ op ziekteverwekkers te herkenen. Complementeiwitten zijn in inactieve vorm aanwezig in het bloed. Als ze in aanraking komen met antilichamen die zijn gebonden aan bacteriën worden ze actief en gaan plakken aan het

complex van antilichaam en bacterie. Geactiveerde complementeiwitten kunnen in sommige gevallen direct de bacterie doden. Daarnaast worden geactiveerde complementeiwitten ook herkend door macrofagen. Als een macrofaag een bacterie met geactiveerde complementeiwitten tegenkomt gaat hij over tot de fagocytose (het opeten) van de bacterie (Figuur 3B). Op die manier werken antilichamen en complement samen om macrofagen aan te zetten tot het opruimen van ziekteverwekkers. Om gebonden antilichamen en complement te herkennen gebruiken macrofagen 'herkenningeiwitten' (receptoren) die op de buitenkant van de macrofaag zitten. Voor antilichamen heten deze herkenningseiwitten **Fcγ receptoren** (zie hieronder), voor complementeiwitten heten ze complementreceptoren.
Tijdens de immuunrespons scheiden zowel T cellen, B cellen als macrofagen chemische componenten uit die niet alleen de ziekteverwekker opruimen, maar ook zorgen voor nog meer actieve witte bloedcellen, die op hun beurt weer kunnen bijdragen aan de immuunrespons. Dit proces heet **ontsteking**.

1.4. Auto-immuniteit bij MS patiënten

Het is ontzettend belangrijk dat het immuunsysteem het verschil weet tussen schadelijke, lichaamsvreemde structuren (virussen en bacteriën) en de structuren die bij het lichaam horen (lichaamseigenstructuren, zoals myeline). Als het immuunsysteem dit onderscheid niet (meer) kan maken ontstaat een **auto-immuunziekte**. Het immuunsysteem (witte bloedcellen, antilichamen en complement) richt zich dan op lichaamseigen structuren, met vaak zeer schadelijke gevolgen. In het geval van MS is de auto-immuunreactie gericht tegen de myeline die de axonen in het CZS omhult. Omdat het immuunsysteem de myeline nu als schadelijk beschouwt, zal het proberen de myeline op te ruimen. Daardoor ontstaan ontstekingsreacties in het CSZ, waarbij myeline wordt afgebroken (demyelinisatie) door geactiveerde T cellen, macrofagen, antilichamen en complement. De chemische componenten die tijdens de onstekingsreactie worden uitgescheiden zijn niet alleen schadelijk voor myeline, maar beschadigen ook de axonen. Deze schade is vaak onherstelbaar, waardoor de verlammingsverschijnselen bij MS patiënten meestal blijvend zijn.
Men vermoedt dat macrofagen een grote rol spelen bij de demyelinisatie in MS, waarschijnlijk door de fagocytose (het opeten) van de myeline. Daarnaast zijn ook **anti-myeline antilichamen** (antistoffen die specifiek plakken aan myeline) en complementeiwitten aanwezig in de hersenen van MS patiënten. De kans bestaat dus dat macrofagen geactiveerd raken door antilichamen en complementeiwitten die zijn gebonden aan myeline. Het is echter niet bekend hoe belangrijk antilichamen en complement zijn voor demyelinisatie. Vooral over de rol van anti-myeline antilichamen in MS bestaan nog veel vragen.

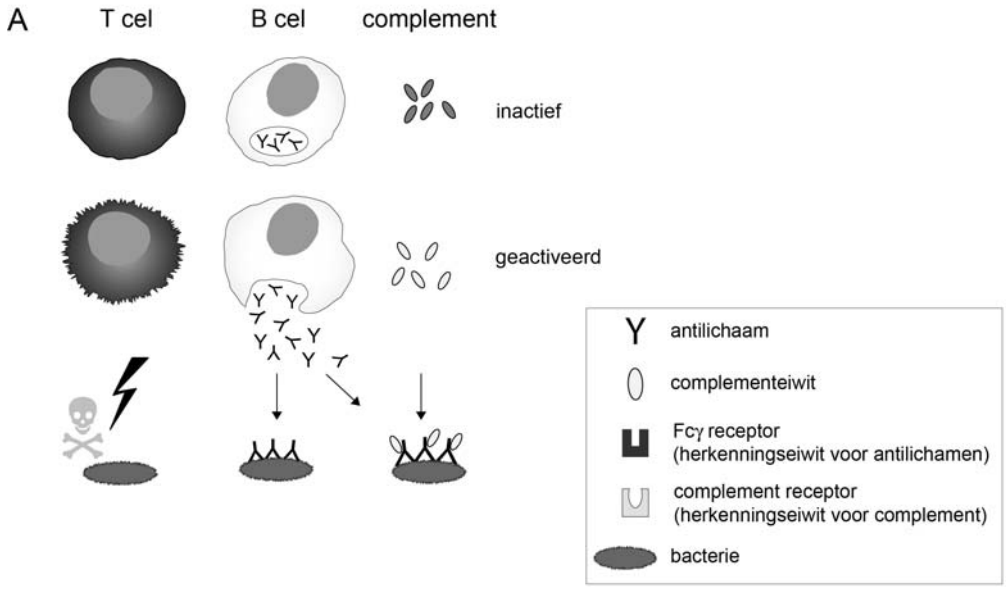
2. Promotieonderzoek

Dit proefschrift beschrijft onderzoek naar de manier waarop antilichamen gericht tegen myeline een bijdrage kunnen leveren aan de onstekingsreactie in MS.

2.1. Antilichamen en complement zijn betrokken bij myeline fagocytose in MS

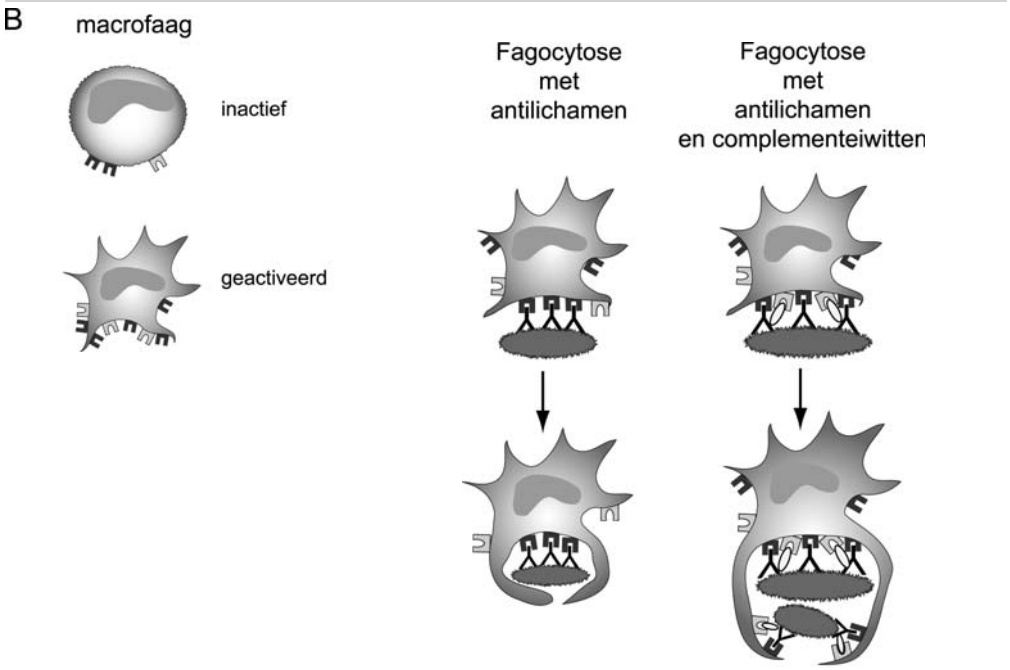
Als anti-myeline antilichamen binden aan myeline, kan dit leiden tot demyelinisatie als de gebonden antilichamen worden herkend door macrofagen. Om gebonden antilichamen te herkennen en te fagocyteren (op te eten), maken macrofagen gebruik van 'herkenningseiwitten', de **Fcγ receptoren**, die op de buitenkant van macrofagen zit. Antilichamen en Fcγ receptoren passen in elkaar als een sleutel in een slot. Een macrofaag die geen Fcγ receptoren heeft, is niet in staat gebonden antilichamen te herkennen. Daarnaast kan demyelinisatie worden veroorzaakt door de activatie van complementeiwitten.

Het is bekend dat antilichamen, macrofagen (met Fcγ receptoren op de buitenkant) en complement aanwezig zijn in de hersenen van MS patiënten, op de plaatsen waar demyelinisatie optreedt (laesies). Het is echter niet duidelijk of antilichamen en complement



Figuur 3A. Witte bloedcellen en complement in inactieve en geactiveerde staat.
T cellen, B cellen, complement en macrofagen circuleren door het lichaam in inactieve staat (bovenste rij). Als ze een ziekteverwekker zoals een bacterie tegenkomen raken ze geactiveerd (tweede rij). T cellen zijn direct in staat bacterien te doden. B cellen zitten vol met antilichamen, die ze na activatie uitscheiden. Die antilichamen plakken aan de bacterie. Complementeiwitten binden weer aan de antilichamen die aan de bacterie plakken.

aanwezig zijn in de laesies van àlle MS patiënten. Omdat het vermoeden bestaat dat het ziekteproces niet in alle MS patiënten hetzelfde is, is de kans aanzienlijk dat antilichamen en complement in slechts een deel van de patiënten een rol spelen.
Om dit te onderzoeken hebben we gebruikt gemaakt van autopsie-hersenmateriaal van MS patiënten. Dit is gebeurd in samenwerking met de Nederlandse Hersenbank, die autopsies van hersendonoren en de uitgifte van hersenmateriaal coördineert. Omdat het niet mogelijk is de hersenen van levende MS patiënten van binnen te bekijken, is onderzoek aan donorhersenen is erg belangrijk voor een beter inzicht in de ziekte MS.
Op dunne plakjes hersenmateriaal kunnen bepaalde structuren (in dit geval bijvoorbeeld antilichamen en macrofagen) zichtbaar gemaakt worden door ze te 'kleuren' met kleurstoffen die specifiek zijn voor de gewenste structuur. Wij hebben dit gedaan met hersenmateriaal van een grote groep MS patiënten en we hebben de 'kleuring' van de hersenen vergeleken met de hersenen van gezonde hersendonoren.



Figuur 3B. Macrofagen 'eten' bacterien met behulp van antilichamen en complement
Macrofagen hebben herkenningseiwitten op de buitenkant (Fcγ receptoren en complementreceptoren) waarmee ze respectievelijk antilichamen en complementeiwitten herkennen. Als er een bacterie in de buurt is raken macrofagen geactiveerd, waardoor er nog meer herkenningseiwitten op de buitenkant komen. Als een macrofaag gebonden antilichamen of complement herkent begint hij te eten (fagocytose). Als aan een bacterie zowel antilichamen als complementeiwitten zijn geplakt eet de macrofaag meer dan als er alleen antilichamen zijn geplakt.

Wij hebben ontdekt dat antilichamen en geactiveerde complementeiwitten aanwezig waren in de laesies van àlle MS patiënten. Omdat antilichamen en complementeiwitten op precies dezelfde plaatsen aanwezig waren (co-localisatie) is het mogelijk dat de complementeiwitten geactiveerd zijn geraakt doordat ze in aanraking kwamen met antilichamen. Daarnaast vonden we talloze macrofagen, met veel Fcγ receptoren op de buitenkant. De belangrijkste bevinding van dit onderzoek was dat antilichamen, samen met FcγR en kleine brokstukjes myeline, te vinden waren binnenin macrofagen. Dit duidt erop dat macrofagen de myeline hebben gefagocyteerd (opgegeten). Binnenin de macrofagen waren ook antilichamen en Fcγ receptoren te zien, dus het lijkt erop dat antilichamen en Fcγ receptoren hebben bijgedragen aan de fagocytose van myeline. In dezelfde macrofagen waren ook complementeiwitten aanwezig. Dit duidt erop dat zowel antilichamen als complementeiwitten een belangrijke rol spelen bij demyelinisatie in MS patiënten (zie ook de kleurenfiguren op pagina 163, 166, 167, 170 en 171).

2.2. Anti-myeline antilichamen in een diermodel voor Multiple Sclerose

Hoewel onderzoek naar autopsie materiaal van MS patiënten erg zinvol is, verschaft het geen informatie over het verloop van de ontstekingsprocessen die bijdragen aan demyelinisatie (een autopsie is immers een momentopname).

Om de rol van antilichamen en Fcγ receptoren op het ontstaan en verloop van MS beter te kunnen onderzoeken hebben we gebruikt gemaakt van proefdieren.

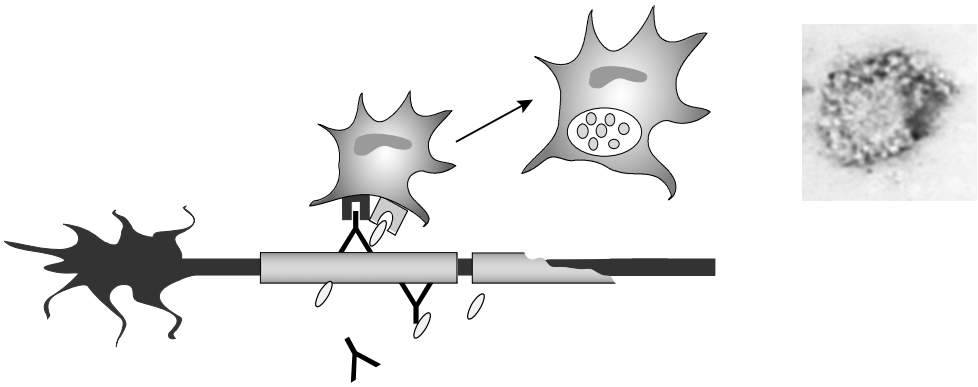
Als muizen worden geinjecteerd met myeline (of componenten van myeline) ontwikkelen ze een auto-immuunreactie tegen myeline, waardoor onstekingen ontstaan in de hersenen, met demyelinisatie en verlamming. Die ziekte die dan ontstaat heet Experimentele Autoimmune Encephalomyelitis (**EAE**) en vertoont grote overeenkomsten met MS.

Om te kijken of Fcγ receptoren belangrijk zijn voor het onstaan van EAE hebben we gebruik gemaakt van Fcγ receptor knockout muizen (**FcRγ$^{-/-}$ muizen**). De macrofagen van deze muizen hebben nauwelijks Fcγ receptoren op hun oppervlakte, en zijn daardoor niet goed in staat gebonden antilichamen te herkennen.

Na injectie met myelinecomponenten raakten alle FcRγ$^{-/-}$ muizen ernstig verlamd. Hieruit blijkt dat Fcγ receptoren (en dus ook de activatie van macrofagen door antilichamen) niet essentieel is voor het ontstaan van EAE.

Uit onderzoek in het verleden is bekend dat anti-myeline antilichamen kunnen bijdragen aan demyelinisatie in EAE, maar pas in een latere fase van de ziekte. Met andere woorden, anti-myeline antilichamen zijn niet belangrijk voor het ontstaan van de ziekte, maar ze kunnen misschien wel een bijdrage leveren aan de voortgang van de ziekte.

Om de rol van anti-myeline antilichamen en Fcγ receptoren in een latere fase van EAE te onderzoeken hebben we EAE geinduceerd in normale muizen en in FcRγ$^{-/-}$ muizen. Nadat ze ziek werden, kregen de muizen een injectie met anti-myeline antilichamen. De verlammingsverschijnselen van EAE werden hierdoor onmiddelijk een stuk ernstiger, zowel in normale als in FcRγ$^{-/-}$. Hieruit blijkt dat Fcγ receptoren niet nodig zijn voor de schadelijke effecten van antilichamen, want de verergering van de ziekte onstaat ook er geen Fcγ



Figuur 4

Antilichamen en complement binden aan myeline in de hersenen van MS patiënten. Macrofagen herkennen nu de myeline met behulp van Fcγ receptoren en complementreceptoren en beginnen de myeline op te eten. Hierdoor verdwijnt de myeline van het axon. Macrofagen die te vinden zijn in de hersenen van MS patiënten zitten soms helemaal vol myeline brokstukjes De foto rechts is een microscopische opnamen van een 'volgegeten'macrofaag in de hersenen van een MS patiënt. Voor kleurenfoto's van myeline, antilichamen, complementeiwitten en Fcγ receptoren in en op macrofagen (in de hersenen van een MS patiënt), zie pagina's 163, 167, 170 en 171.

receptoren niet aanwezig zijn. Het is waarschijnlijk dat het acute effect van anti-myeline antilichamen in EAE wordt veroorzaakt door de andere route waarmee anti-myeline antilichamen schade kunnen aanrichten, via de activatie van complementeiwitten.
Gemiddeld drie weken nadat de muizen waren ingespoten met anti-myeline antilichamen hebben we de hersenen nader onderzocht door ze in dunne plakjes te snijden en ze te bestuderen onder de microscoop. In de hersenen van normale muizen was ernstige demyelinisatie te zien, terwijl de demyelinisatie bij FcRγ$^{-/-}$ muizen gering was. Dus, ookal waren FcRγ$^{-/-}$ receptoren niet nodig voor acute verergering van de EAE door anti-myeline antilichamen, op langere termijn lijken FcRγ$^{-/-}$ receptoren toch een bij te dragen aan demyelinisatie (voor een kleurenfoto van demyelinisatie in de hersenen van normale muizen, zie pagina 162).

2.3. De invloed van genetische verschillen in Fcγ receptoren op MS

De Fcγ receptoren, die op de buitenkant van macrofagen zitten, zijn niet bij iedereen hetzelfde. Dit is het gevolg van een **genetisch polymorfisme**, een vorm van genetische variatie tussen mensen, die normaal gesproken geen invloed heeft op de gezondheid. Een bekend voorbeeld van een genetisch polymorfisme zijn de bloedgroepen (A, B, AB en O).
Door genetische polymorfismen in Fcγ receptoren, is de binding tussen antilichamen en Fcγ receptoren niet bij alle mensen even efficiënt, met als gevolg dat macrofagen minder goed in staat zijn gebonden antilichamen te herkennen. Uit onze studie in autopsie hersenen bleek dat macrofagen in de hersenen van MS patiënten veel Fcγ receptoren op de buitenkant

hadden, en uit onze studie in muizen bleek dat Fcγ receptoren mogelijk op lange termijn bijdragen aan demyelinisatie. Het is daarom mogelijk dat MS patiënten met bepaalde genetische polymorfismen in hun Fcγ receptoren minder gevoelig zijn voor antilichaam-gemedieerde demyelinisatie waardoor de ziekte langzamer verloopt. Om dit te onderzoeken hebben we van 432 MS patiënten en 515 gezonde donoren genetisch materiaal (DNA) afgenomen om te kijken welke typen Fcγ receptoren aanwezig waren. Bij gezonde donoren en MS patiënten kwamen dezelfde typen Fcγ receptoren voor, wat erop wijst dat de kans op het ontstaan van MS niet beinvloedt wordt door genetische polymorfismen in Fcγ receptoren. Daarnaast bleek het ziekteverloop bij MS (de snelheid waarmee een patiënt achteruit gaat) niet gecorreleerd te zijn met genetische polymorfismen in Fcγ receptoren.
Dit zou kunnen betekenen dat Fcγ receptoren echt geen rol spelen bij MS. Het is ook mogelijk dat Fcγ receptoren alleen belangrijk zijn in een bepaalde groep MS patiënten, namelijk de MS patiënten die veel anti-myeline antilichamen hebben. Helaas is het onduidelijk bij welke patiënten dit het geval is, en we kunnen die patiënten dus niet apart testen.
In het laatste deel van mijn promotieonderzoek hebben we een test ontwikkeld, die kan bepalen of MS patiënten anti-myeline antilichamen in het bloed hebben.

2.4. Anti-myeline antilichamen in het bloed van MS patiënten

Als anti-myeline antilichamen inderdaad een rol spelen bij (een deel van de) MS patiënten is de kans groot dat anti-myeline antilichamen ook in het bloed van die patiënten zijn te vinden. Om dit te onderzoeken is op de afdeling Moleculaire Celbiologie en Immunologie een test ontwikkeld die antilichamen detecteert die speciaal gericht zijn tegen myeline.
Om te testen of zulke antilichamen aanwezig zijn in het bloed van MS patiënten, hebben we bloed afgenomen van 160 MS patiënten en 40 gezonde mensen. Daarin hebben we de hoeveelheid anti-myeline antilichamen gemeten.
Ongeveer de helft van de MS patiënten bleek een verhoogde hoeveelheid anti-myeline antilichamen in het bloed te hebben, terwijl de andere helft van de MS patiënten geen verschil vertoonde met gezonde donoren. Dit kan betekenen dat anti-myeline antilichamen een rol spelen in ongeveer de helft van de MS patiënten.

2.5. Conclusie

Het lijkt erop dat anti-myeline antilichamen betrokken zijn bij demyelinisatie in MS. Zowel de activatie van macrofagen via Fcγ receptoren als activatie van complementeiwitten zijn hierbij betrokken. Antilichamen en complement waren aanwezig in de hersenen van alle MS patiënten die door ons zijn bestudeerd, hoewel slecht vijftig procent van de MS patiënten ook anti-myeline antilichamen in het bloed had. Het is daarom niet duidelijk of anti-myeline antilichamen bij alle patiënten van belang zijn.
Als duidelijk is bij welke patiënten anti-myeline antilichamen schadelijk zijn, wordt het mogelijk speciaal voor die patiënten een medicijn te ontwikkelen die gericht is op anti-myeline antilichamen. Dit zou een grote vooruitgang betekenen, omdat de medicijnen die nu worden gebruikt voor de behandeling van MS niet altijd effectief zijn en veel bijwerkingen hebben.

Color figures

Chapter 3:

Chapter 5:

Chapter 3, figure 4 (page 61)

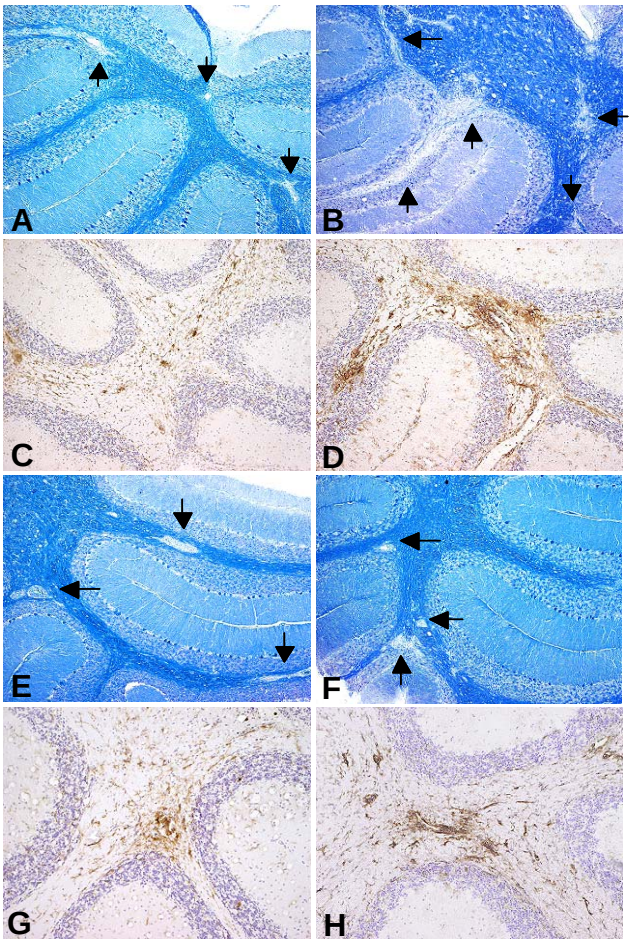


Figure 4.

CNS inflammation and demyelination in anti-MOG antibody exacerbated EAE in Wt and FcRγ$^{-/-}$ mice. Brains were isolated 35 days after immunisation, and the cerebellum white matter was analysed for demyelination using Kluver-Barrera staining (A,B,E,F) and inflammation using mac-1 staining (C,D,G,H). In Wt mice, injection of Z12 mAb resulted in enhanced demyelination (B) and inflammation (D) when compared to saline-injected animals (A and C respectively). In FcRγ$^{-/-}$ mice, injection of Z12 mAb did not change demyelination (F) or inflammation (H) compared to saline-treated mice (E,G). Areas of demyelination are indicated by arrows. Original magnification 40x. (I) Quantitative analysis of macrophage infiltration (percentages calculated as [mac-1-positive area/total cerebellum white matter area] x 100). Data represent average of 7 mice (Wt-NaCl; FcRγ$^{-/-}$-NaCl), 2 mice (Wt-Z12 mAb) or 3 mice (FcγR$^{-/-}$-Z12 mAb).

Chapter 5, figure 1 (page 93)

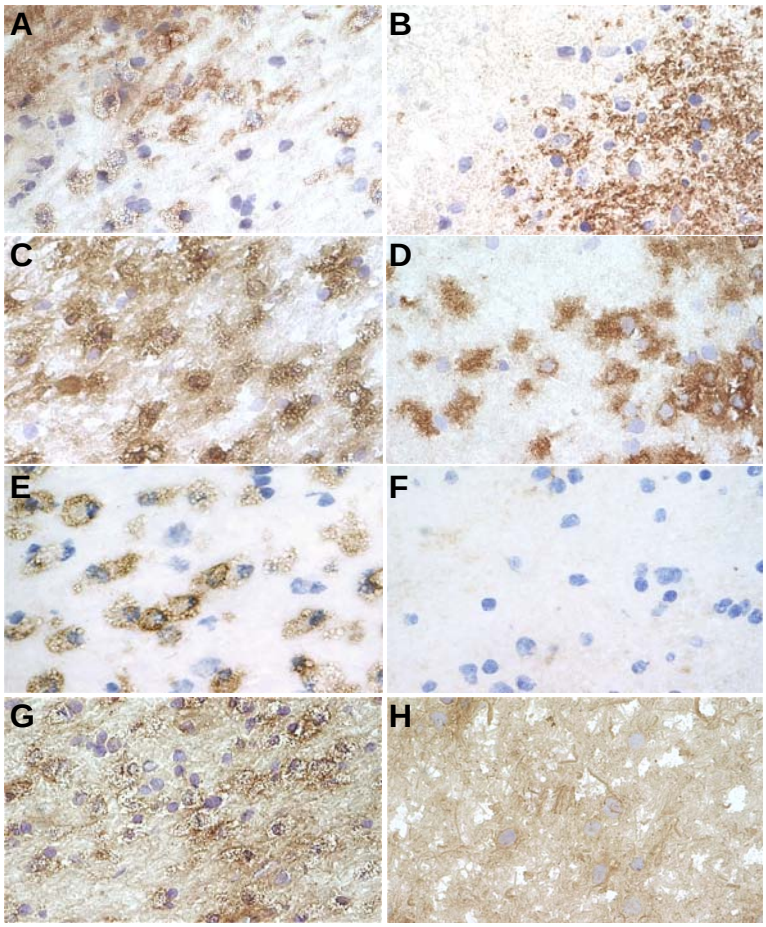


Figure 1.
Complement activation products and IgG are present on and within macrophages in inflammatory demyelinating lesion areas. Left panel: inflammatory demyelinating area; right panel: inflammatory non-demyelinating area. Both areas were located at the border of a chronic active MS lesion. Macrophages in inflammatory demyelinating areas contain intracellular PLP (A) whereas macrophages in inflammatory non-demyelinating areas do not (B). Macrophages in both inflammatory demyelinating and non-demyelinating areas express high levels of HLA-DR (C, D). Expression of C5b-9 is associated with macrophages in inflammatory demyelinating areas (E) but not inflammatory non-demyelinating areas (F). Diffuse immunostaining for IgG, and IgG staining associated with astrocyte processes is observed in demyelinating (G) and non-demyelinating (H) lesion areas, but staining on and within macrophages is restricted inflammatory demyelinating areas (G).

Chapter 5, figure 3A-C (page 95)

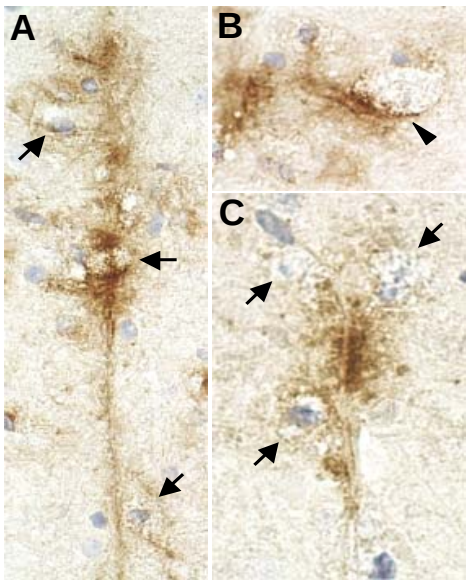


Figure 3A-C.
C3d and C4d are detected on myelin sheaths in inflammatory demyelinating areas. C3d immunostaining is observed on a myelin sheath that is in close contact with macrophages (A, macrophages indicated by arrows), immunostaining is more intense at the interface of the myelin sheath and the macrophage (B, interface indicated by arrowhead). C4d immunostaining on a myelin sheath that is surrounded by macrophages (C, macrophages indicated by arrows). Original magnification 400X. (D) Results of semi-quantitative analysis of myelin associated immunostaining for C3d and C4d. The percentage of areas that were positive for complement is represented by the height of the bars, whereas the shading of the bars represents the extent of immunopositivity.(+) light immunopositivity, (++) moderate immunopositivity and (+++) strong immunopositivity.
**immunostaining higher than in inactive lesions and NAWM ($p<0.05$).

Chapter 5, figure 4 (page 96)

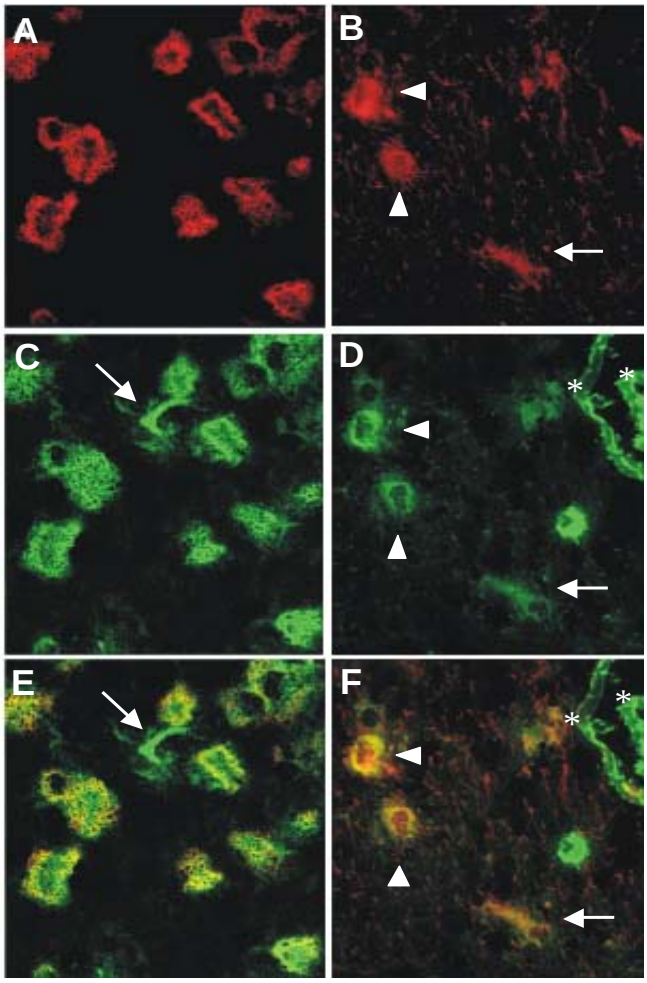


Figure 4.

Double labelling immunofluorescence reveals colocalisation of PLP with complement and complement with IgG in inflammatory demyelinating areas. The left panel shows colocalisation of PLP (A) and C3d (C) on the surface and in vesicle-like structures inside phagocytic macrophages (E shows merge of A and C). Immunostaining for C3d, but not PLP, is observed on astrocytes (arrow). The right panel shows colocalisation of C1q (B) with IgG (D) on infiltrating phagocytic monocytes/macrophages (arrowheads) and on a glial cell (arrow) (F shows merge of B and D). Vascular immunostaining for IgG, but not C1q, is observed on the inner and outer basement membranes lining the Virchow-Robin space (D, F, asterix). The IgG-positive, C1q-negative cell on the left side of image D and F may represent a plasma cell. Original magnification 400X.

Chapter 5, figure 6A-D (page 98)

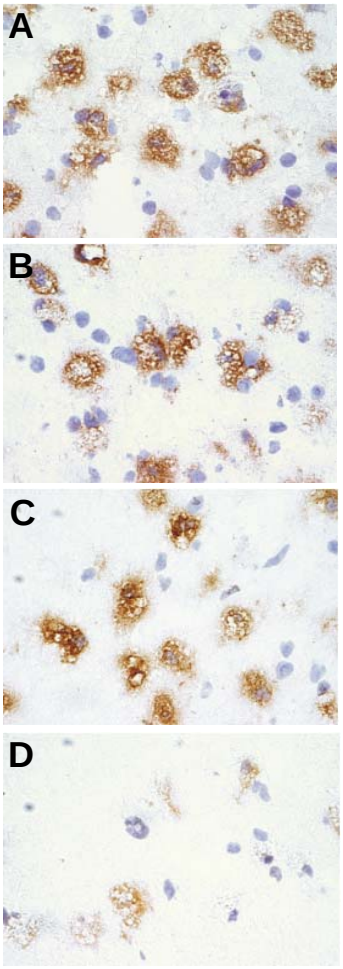


Figure 6A-D.

High expression of Fcγ R in inflammatory areas in chronic MS. Figure shows abundant expression of HLA-DR (A) on phagocytic macrophages in an inflammatory demyelinating area at the border of a chronic active lesion. In the same area, high expression of Fcγ RI (B) and Fcγ RII (C) is detected on the surface of phagocytic macrophages and within intracellular vesicle-like structures. Expression of Fcγ RIII (D) is observed in association with phagocytic macrophages as well, although the extent of immunostaining is lower than for Fcγ RI and Fcγ RII. Original magnification 400X. (E) Results of semi-quantitative analysis of Fcγ R in different lesion areas. The percentage of lesion areas that were positive for Fcγ R expression is represented by the height of the bars, whereas the shading of the bars represents the extent of immunopositivity. (+) light immunopositivity, (++) moderate immunopositivity and (+++) strong immunopositivity.

**immunostaining higher than in inactive lesions and NAWM ($p<0.05$), *immunostaining higher than NAWM ($p<0.05$).

Chapter 5, figure 7 (page 99)

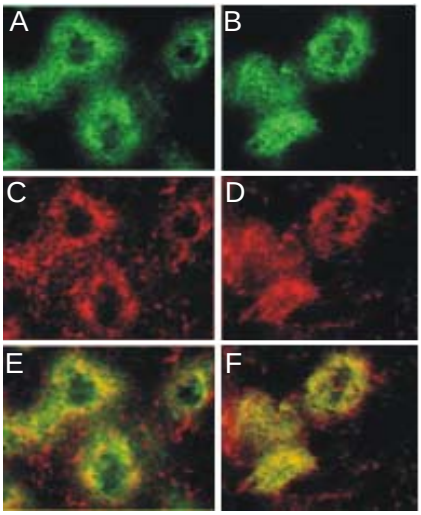


Figure 7.

Double labelling immunofluorescence reveals colocalisation of FcγR with IgG and complement an inflammatory demyelinating area at the border of a chronic active MS lesion. The upper panel shows colocalisation of FcγRII (A) and IgG (C) on the surface and in vesicle-like structures inside phagocytic macrophages (E shows merge of A and C). The lower panel shows colocalisation of FcγRII (B) with C1q (D) on and within phagocytic macrophages (F shows merge of B and D). Diffuse background staining for IgG (C) and C1q (D) is typical of inflammatory demyelinating areas in the CNS. Original magnification 400X.

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2. Wållberg M, Bergquist J, Achour A, Breij E, Harris RA. Malondialdehyde mofication of myelin oligodendrocyte glycoprotein leads to increased encephalitogenicity and vaccine efficacy. 2005. *Submitted*
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5. Brink BP, Veerhuis R, Breij EC, van der Valk P, Dijkstra CD, Bö L. The pathology of multiple sclerosis is location-dependent: no significant complement activation is detected in purely cortical lesions. *J Neuropathol Exp Neurol* 2005; 64: 147-155.
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7. Breij EC, van der Pol WL, van WL, Jansen MD, Dijkstra CD, van de Winkel JG, Uitdehaag BM. No association of Fc gamma RIIa, Fc gamma RIIIa and Fc gamma RIIIb polymorphisms with MS. *J Neuroimmunol* 2003; 140: 210-215.
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11. van der Smagt MJ, Breij EC, van de Grind WA. Spatial structure, contrast polarity and motion integration. *Vision Res* 2000; 40: 2037-2045.

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